The evaluation and validation of Phadebas® paper as a presumptive screening tool for saliva on forensic exhibits

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Declaration

I declare that this thesis does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due reference has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Danielle J. Wornes

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PART 1

Literature Review

REVIEW

The evaluation and validation of Phadebas® paper as a presumptive screening tool for saliva on forensic exhibits

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Abstract

Biological evidence originating from saliva can be of considerable significance to criminal investigations. However, the localisation of saliva is inherently challenging, due to the fact that saliva, and its associated stains, do not contain readily visible constituents. Under such circumstances, it is necessary to adopt screening techniques in order to isolate potential saliva stains. There are a limited number of screening tools available for this purpose; currently, the state DNA forensic laboratory of Western Australia (PathWest) utilises alternate light source technology. However, this method has demonstrated ineffectiveness, with a fail rate of 40% or greater. Thus, Phadebas® paper has been suggested as an alternative. This test detects the activity of the α-amylase enzyme, which is present in high concentrations in saliva. The limitations of Phadebas® paper for use as a presumptive screening tool need to be understood for the correct interpretation and inclusion of test results in forensic investigations. Thereby, the following review aims to assess the suitability of Phadebas® paper as a presumptive screening test for saliva. In this review, current screening techniques for saliva have been examined, as well as the reported sensitivity and specificity of Phadebas® paper. Furthermore, the factors affecting sensitivity and specificity have been explored. The use of Phadebas® paper in the greater context of forensic examination has also been considered. Finally, suggestions have been made pertaining to experimental design and methodology for studies concerning this topic; recommendations for future studies have also been advised. This review found that the limitations of Phadebas® paper as a presumptive screening tool are poorly reported and attempts to validate this test lack scientific rigour. Consequently, five crucial areas have been identified for consolidation; specifically, the sensitivity, specificity, effects of temperature on sensitivity and specificity, detection of saliva in mixed body fluid stains, and influence of the substrate on Phadebas® paper need to be subjected to experimental validation.

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1 Introduction

The evidence associated with the detection and identification of saliva in criminal investigations can be of considerable significance. The existence of saliva at a crime scene or on an exhibit is a valuable source for establishing physical presence or interaction as well as determining movement within a scene (Maloney & Housman, 2014). In being able to effectively detect and identify saliva, samples can be processed and prepared for subsequent forensic testing; most significantly, such samples can be used in deoxyribonucleic acid (DNA) analysis for human identification purposes. The information obtained can then be presented in legal proceedings to corroborate or refute statements given by the accused and injured party. However, the forensic significance of saliva is often understated. This is reflected in the literature, with techniques for the detection and identification of human biological evidence largely focussing on blood and semen (Gunn, 2009; Houck & Siegal, 2011), which are considered the most commonly encountered body fluids at crime scenes. Conversely, a study by Bond and Hammond (2008) found that the primary source of DNA evidence in volume crime in the United Kingdom arises from saliva, typically located on peripheral items such as drinking vessels and cigarette ends. Furthermore, establishing the nature of, and involvement in, a physical or sexual assault may rely on the detection of saliva on exhibits such as swabs, clothing or bedding from actions such as biting, saliva expectoration or oral intercourse (Breathnach & Moore, 2013, 2015). Regardless of the means by which saliva becomes deposited at crime scenes or on exhibits, the practising forensic scientist is faced with one fundamental issue: the detection (or more specifically the localisation) of saliva.

The localisation of saliva during forensic examination is often challenging unless targeted sampling can occur, such as on areas where saliva is highly likely to exist, i.e. on the mouth of drinking vessels and the filter ends of cigarettes. When the location of a saliva deposit is not so predictable,

its presence can remain undetected, since saliva, and its associated stains, lack readily visible constituents. In fact, whole saliva is composed of >99% water with the remaining <1% consisting of proteins, glycoproteins (enzymes and antibodies), and electrolytes (Gunn, 2009; Humphrey & Williamson, 2001). Since saliva stains are inherently difficult to observe with the naked eye, it is necessary to adopt screening techniques to attempt visualisation. Alternate light sources (ALS) are typically used in the initial examination of an exhibit due to their non-destructive nature. ALS at 450 nm viewed through an orange filter is generally considered the most useful for observing fluorescence of saliva, however, it is difficult to distinguish between stains originating from saliva and those from other body fluids (Gunn, 2009; Vandenberg & van Oorshot, 2006)). ALS is currently the screening tool utilised in the state DNA forensic laboratory of Western Australia (PathWest) for attempting to locate saliva on exhibits, however, it is not presumptive for saliva and as a consequence all stains located have to be subjected to confirmatory testing with Rapid Stain Identification Saliva (RSID™-Saliva). This is a time-consuming and costly process, especially when large exhibits with multiple body fluids present are encountered. In an attempt to overcome the issues associated with using ALS as a screening tool, it has been proposed that Phadebas® paper (Magle Life Sciences, Lund, Sweden) may be implemented into the forensic laboratory.

Detailed knowledge and validation of the limitations of Phadebas® paper is crucial for the correct interpretation and inclusion of test results into forensic investigations, and thus this test needs to be subjected to intense scrutiny prior to consideration for implementation into the PathWest forensic laboratory. Thereby, the following review aims to assess the suitability of Phadebas® paper as a presumptive screening test for saliva on forensic exhibits. In order to accomplish this, current screening techniques for saliva will be examined and their various advantages and disadvantages will be highlighted. Furthermore, the sensitivity and specificity of this test (and the various factors that may affect sensitivity and specificity) will be thoroughly investigated. Finally, the use of

Phadebas® paper in the greater context of an integrated forensic examination will be considered. Ultimately, the conclusions of this review should direct decisions pertaining to experimental design, and identify areas of interest for future research.

1.1 Composition of unstimulated saliva

In order to understand the logic behind the preferred molecular target for the presumptive testing of saliva, it is useful to appreciate the various elements that constitute this fluid. For the purpose of this investigation, saliva will be defined as the mixture of epithelial cells and secretions originating from more than one source within the oral cavity. The major and minor salivary glands contribute to the bulk of secretions that constitute saliva (Ross & Pawlina, 2010). A smaller amount is derived from gingival sulci, tonsillar crypts, oro- and naso- pharyngeal secretions, and transudate from the epithelial lining of the oral cavity (Aps & Martens, 2005; Chiappin, Antonelli, Gatti, & Palo, 2007). In addition to this, saliva may contain blood-derived compounds (erythrocytes, leukocytes, plasmatic proteins), food debris, chemical products from medications and other drugs, bacteria and fungi (and their metabolites), viruses, and gastrointestinal reflux products (Aps & Martens, 2005; Chiappin et al., 2007; Humphrey & Williamson, 2001).

Aforementioned water constitutes >99% of saliva with the remaining element comprising of organic and inorganic compounds, summarised in Table 1. Significant amounts of some inorganic compounds are present in saliva, particularly potassium and chloride; the primary organic constituents of saliva are α-amylase and lysozyme, which are both enzymes. Thus, since α-amylase is one of the larger components, techniques for the detection and identification of saliva have advanced to utilise this enzyme, specifically its activity (Virkler & Lednev, 2009). Mueller was the first medico-legal investigator to suggest the use of the α-amylase as a basis for identifying saliva

stains in 1928 (Gaensslen, 1983). Furthermore, Nelson and Kirk (1963) confirmed that α-amylase was the most suitable indicator for the presence of saliva, due to its abundance and persistence in saliva stain, when compared to alkaline phosphatase, nitrite and thiocyanate.

Table 1: Composition of unstimulated saliva.

		Concentration - mean (mg/mL)
Organic constituents	Protein	220.0
	α-amylase	38.0
	Lysozyme	22.0
	Urea	20.0
	Secretory immunoglobulin A (sIgA)	19.0
	Cholesterol	8.0
	Cyclic adenosine monophosphate (cAMP)	7.0
	Epithelial growth factor (EGF)	3.4
	Mucin	2.7
	Uric Acid	1.5
	Immunoglobulin G (IgG)	1.4
	Hystatin	1.2
	Glucose	1.0
	Albumin	0.6
	Immunoglobulin M (IgM)	0.2
	Creatinine	0.1
	Cystatins	0.07
	Slatherin	0.04
	Lactoferrin	0.03
	Transferrin	0.006
Inorganic constituents	Potassium	80.0
	Chloride	50.0
	Phosphate	16.8
	Sodium	15.0
	Thiocyanate (smokers)	9.0
	Ammonia	6.0
	Calcium	5.8
	Thiocyanate (non-smokers)	2.0
	Bicarbonate	0.3
	Magnesium	0.005
	Fluoride	Traces (according to intake)

1.2 Amylases

In addition to α-amylase (EC 3.2.1.1), two other types of amylases exist; namely β-amylase (EC 3.2.1.2) and γ-amylase (EC 3.2.1.3). Aside from possessing biological functions, amylases are used

in many industries including the textile, detergent, paper and food industry as well as being crucial to brewing and fermentation processes. All function to catalyse the hydrolysis of starch, glycogen and related polysaccharides and oligosaccharides into smaller carbohydrate molecules but differ in the mode by which they digest starch. The α-amylase enzyme functions to catalyse the hydrolysis of α-1,4-glucosidic linkages in starch, glycogen and related polysaccharides, and oligosaccharides in a random manner. β- and γ- amylase, on the other hand, can only degrade starch from the non-reducing end of the polymer chain by hydrolysing the second and last α-1,4-glucosidic linkage, respectively. γ-Amylase can additionally hydrolyse α-1,6-glucosidic linkages. Due to the differences in mode of digestion, α-amylase digests starch more rapidly compared to the other amylases (Magle Life Sciences, 2014). α-Amylase is present in human, animals, plants, fungi and bacteria, while β-amylase is found only in plants, bacteria and fungi; γ-amylase is less frequently encountered in nature. Since the α-amylase enzyme is the only amylase present in humans, presumptive tests for human saliva have typically adopted enzymatic reaction techniques involving α-amylase and a starch-dye complex.

In humans, the α-amylase enzyme functions to digest dietary starch in the oral cavity. It is expressed by two separate genetic loci on chromosome 1 resulting in the manifestation of two structurally different isoforms, namely salivary and pancreatic α-amylase. The structural difference between these two isoforms is negligible; their amino acid sequence is 97% homologous (Lorentz, 1998; Nishide, Emi, Nakamura, & Matsubara, 1984). Salivary α-amylase is the major enzyme component, and is present in high concentrations, in saliva. However, α-amylases (salivary and pancreatic) are found in other body fluids, including blood, breast milk, faeces, nasal secretions, perspiration, semen, tears, urine vaginal secretions (Fridhandler, Berk, Montgomery, & Wong, 1974; Huguet, Cortes, Arranz, & Fuentes-Arderiu, 1993; Merritt, Rivas, Bixler, & Newell, 1973; Moriyoshi, Takeuchi, Shiratori, & Watanabe, 1991; Okabe, Uji, Netsu, & Noma, 1984). To

complicate matters further, α-amylase is a ubiquitous enzyme and has remained relatively unchanged throughout the course of evolution. Thus, α-amylase in humans is not dissimilar to that in other animals, plants, bacteria or fungi. The seemingly abundant nature of α-amylase casts considerable doubt on the use of this enzyme as the foundation of a presumptive test, hence the need to thoroughly investigate the sensitivity and specificity of any presumptive test based on the activity of this enzyme, especially as it relates to Phadebas® paper.

1.3 Phadebas® chemistry

Phadebas® chemistry, like all other presumptive tests for saliva, is based on the activity of the enzyme α-amylase (Gaensslen, 1983; Virkler & Lednev, 2009). All Phadebas® products use homogeneously interlinked starch polymers, that are water-insoluble, covalently bound to a blue dye. The blue dye is water-insoluble when bound to the polymers, however, in the presence of α-amylase the polymers are digested and the blue dye becomes water-soluble (Figure 1).

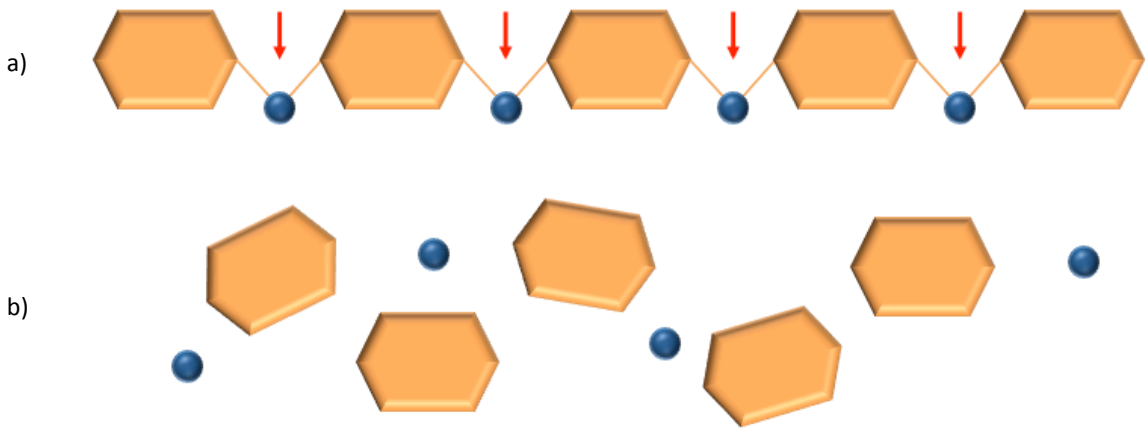

Figure 1: Digestion of Phadebas® starch polymer-dye complex with α-amylase. a) Hexagons and circles represent the starch polymer covalently bound to the blue dye, respectively; arrows indicate the site of action for the α-amylase enzyme. b) Digested starch polymer and the liberation of blue dye molecules.

Two tests are available that utilise this chemistry; the Phadebas® Forensic Tube Test and the

Phadebas® Forensic Press Test, the latter uses Phadebas® paper. The Phadebas® Forensic Tube test is considered a semi-quantitative method for the detection of saliva and uses Phadebas® Amylase Test tablets, however, is only useful for the presumptive testing of suspicious stains, i.e. stains that have already been located. Once the α-amylase in a given sample digests the starch polymer-dye complexes in solution, the resulting blue colour can be read semi-quantitatively on a spectrophotometer; alternatively, a blue coloured solution indicates a positive result. Phadebas® paper is solely a qualitative method and uses starch polymer-dye complexes that are immobilised onto filter paper; when the blue dye is liberated (as a result of α-amylase digestion) it diffuses through the pores of the filter paper, resulting in the localisation of α-amylase positive areas on exhibits (Figure 2). In this sense, Phadebas® paper has capacity as both a presumptive test, and localising screening tool for saliva stains; this is particularly useful for large exhibits such as bedclothes. The Phadebas® Forensic Press Test requires a relatively quick 40-minute examination period, which produces qualitative results in the form of colour visualisation (Figure 2).

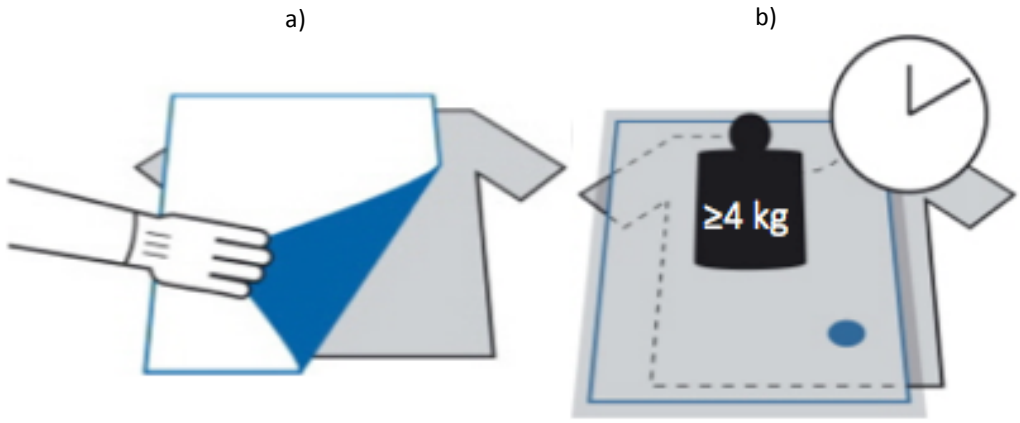

Figure 2: Detection of α-amylase positive areas on forensic exhibits. a) The item under examination is placed on a clean flat surface and dampened with distilled water. Phadebas® paper is placed over the area to be tested (blue reagent side down) and dampened with distilled water. b) An outline of the area being tested is traced onto the Phadebas® paper and a clean glass board is placed on top with a 4 kg weight (or heavier). A timer is started and observations are noted intermittently over the course of 40 minutes.

Phadebas® paper in its pre-manufactured from has only been available for the past 10 years. Prior

to the launch of Phadebas® paper in 2007, forensic laboratories were inventive in creating in-house Phadebas® paper by suspending the tablets in an agar gel plates or dissolving Phadebas® tablets (available since the 1970s) in water and spraying the solution onto blotting or filter paper; Willott (1974) was the first to apply a dyed starch substrate for the forensic identification of saliva using the Phadebas® agar gel plate approach. To be clear, this investigation is solely concerned with the possible implementation of the pre-manufactured Phadebas® paper and not the in-house version, since standardisation and quality control is integral to modern day forensics and this is managed by the manufacturer with Phadebas® paper.

2 Other screening techniques for saliva vs. Phadebas® paper

Only a small number of screening and presumptive tests have been developed for the detection of saliva; an even smaller portion of these have the ability to both act as a presumptive test and screening tool for saliva stains. In the following section, the various advantages and disadvantages in using ALS and enzymatic techniques (starch-iodine radial diffusion test and red-starch paper) for the detection of saliva stains will be outlined and compared to Phadebas® paper (also an enzymatic method).

2.1 Alternate light source techniques

Although ALS is not presumptive for saliva, it is reviewed here since it is the method adopted in the PathWest forensic laboratory for the screening of saliva. Aforementioned, ALS is a simple and non-invasive screening technique – the advantages of ALS as a screening tool for saliva do not extend much further beyond this statement. Aside from experiencing complications in discriminating between saliva and semen (Gunn, 2009; Vandenberg & van Oorshot, 2006), saliva is also naturally harder to detect due to the lack of solid particles in the sample (Jones, 2005) and as a consequence the fluorescence observed is typically weaker (Fiedler et al., 2008; Miranda, Prado, Delwing, & Júnior, 2014). Since semen and saliva are likely to be present together on exhibits (i.e. in sexual assaults), ALS is of little use and is highly likely to produce false positive results. Furthermore, the sensitivity of this technique to saliva stains is concerning. Fiedler et al. (2008) described the detection of saliva using the Lumatec® superlight 400 mercury-arc lamp (at a range of 415–490 nm) to be accurate in only 60% of cases, meaning that 40% of saliva stains remained undetected. This particular ALS is considered to be highly effective for the detection of saliva stains; Fiedler et

al. reported higher detection rates than what was described in Auvdel (1987) who found that 30% of saliva stains tested were detectable with a Spectra-Physics Model 171-19 argon ion laser, while 21% were detectable with ultraviolet (UV) light sources (Mineralight® multiband ultraviolet lamp and Fotodyne Foto UV 410). Therefore, the extent to which saliva stains are persisting unobserved on exhibits in forensic laboratories may extend beyond this figure. Additionally, the colour of the substrate upon which saliva stains (or any body fluids) are deposited can lower the detection rate further with dark coloured substrates impeding the visibility of saliva stains (Fiedler et al., 2008). These poor results with ALS reinforce the need for a more sensitive and specific technique for the localisation and identification of saliva.

The overall detection rate of Phadebas® paper is higher than that of ALS. Hedman et al. (2008) carried out a comparison between Phadebas® paper and ALS from four different manufacturers for the detection of saliva; CrimeScope® CS-16-10 (SPEX Forensics), Quaser 2000/30™ (Attestor Forensics), UV spotlight (Labino) and Polilight® PL6 (Rofin). In this study, the Phadebas® paper resulted in a higher detection rate of saliva on cotton, leather, and untreated and painted wood, with the exception being suede. In contrast, Vandenberg and van Oorschot (2006) concluded that the Polilight® (PL 500 (Rofin)) was as good as Phadebas® paper for screening for potential saliva stains. However, this study was undertaken prior to 2007, i.e. before the release of pre-manufactured Phadebas® paper. As such, in-house Phadebas® paper was produced by dissolving 0.9 g of Phadebas® tablets in 100 mL of distilled water and subsequently spraying the solution onto a sheet of blotting paper. However, the methods adopted by Vandenberg and van Oorschot (2006) differed greatly to that provided by the manufacturer at the time, which stated that 50 tablets should be dissolved in 200 mL of distilled water; they used the equivalent of only 4 or 5 tablets in 100 mL of distilled water, which explains the apparent equality in detection rates of ALS compared to Phadebas® paper. This study and the associated conclusions are methodologically invalid and

should not be included when comparing ALS against Phadebas® paper for the detection of saliva. Furthermore, due to the limited number of valid studies undertaken on this topic, it can be suggested that future research should be conducted, comparing Phadebas® paper and presently available ALS products, for a current opinion.

2.2 Enzymatic techniques

All enzymatic techniques for the detection of saliva are based on the activity of the enzyme α-amylase. However, there are only a limited number of these techniques that have been described for use as a presumptive screening tool for saliva, namely the starch-iodine radial diffusion test, red-starch paper and Phadebas® paper.

2.2.1 Starch-iodine radial diffusion test

The starch-iodine radial diffusion test as a presumptive screening technique for saliva is time-consuming and imprecise; in that, it cannot isolate exact locations of suspected saliva stains. This method was described by Roda et al. (2014), as a presumptive screening method used in the Santa Clara County Crime Lab, whereby multiple swabs were collected from sectioned areas on the exhibit under examination. Saliva was subsequently extracted from these samples, which were then placed in a gel test plate containing starch and left to incubate, generally 6 hours to overnight. Iodine was then added to stain the plate; a positive indication for α-amylase activity was observed as a clear ring on the plate where the saliva extract was deposited. In addition to taking nine times longer (minimum) to obtain results compared to Phadebas® paper, this technique is prone to yielding false positive results in the presence of competing proteins such as albumin and gamma-globulin, which occur naturally in blood and semen (Wilding, 1964). It is obvious that this method is

neither practicable nor efficient as a presumptive screening tool for saliva in the forensic laboratory.

2.2.2 Red-starch paper

Red-starch paper functions on the same principle to that of Phadebas® paper and was created in response to the high demand and lack of availability of Phadebas® tablets, used to create in-house Phadebas® paper. Traditionally, red-starch paper used Procion® Red MX-2B-amylocpectin as the starch-dye complex (Whitehead & Kipps, 1975), however, this reagent is no longer available (Martin, Clayson, & Scrimger, 2006). More recently, Procion® Red MX-5B-partially depolymerised starch for the detection of α-amylase activity has been used (Martin et al., 2006; Vennemann, Scott, Curran, Bittner, & Tobe, 2014). However, red-starch paper has to be produced in-house prior to use, raising concerns regarding standardisation, or lack thereof. As demonstrated with the Vandenberg and van Oorschot study (2006), the lack of using a uniform procedure for the production of in-house paper has significant ramifications; in that, it can lead to misinterpretation of the results and the provision of misinformation to the greater scientific community. If red-starch paper was pre-manufactured these implications would no longer exist and could potentially rival the use of Phadebas® paper. The limitations with respect to the sensitivity and specificity of red-starch paper have not thoroughly been investigated, with only two studies attempting to evaluate the use of it as a presumptive screening tool for saliva (Martin et al., 2006; Vennemann et al., 2014).

3 Sensitivity and specificity of Phadebas® paper

Throughout this review, it has been reiterated that the validation of the sensitivity and specificity of Phadebas® paper is integral to its implementation into any forensic laboratory. Despite the appeal of Phadebas® paper as a presumptive screening tool for saliva, there have been limited studies conducted validating Phadebas® paper for this purpose. The following information investigates past studies reporting the sensitivity and specificity of Phadebas® paper.

3.1 Reported sensitivity of Phadebas® paper

According to the manufacturer, to assure selectivity for saliva, the Phadebas® paper must not detect stains with α-amylase activity below 2000 international units per litre (U/L) within the 40-minute testing window (Magle Life Sciences, 2014). Studies attempting to validate the sensitivity of Phadebas® paper, typically report detection limits in terms of a dilution factor, neglecting considerations of the starting α-amylase activity of the sample. As a result the sensitivity in terms of the lower detection limit has been reported within a wide range of 1:100–1:1000 (Table 1). It is apparent that these values are of little significance and interpretation of the sensitivity is impossible without knowledge of the starting α-amylase activity within saliva samples. Therefore, to provide relevance and to be able to validate the sensitivity of Phadebas® paper in concordance with the manufacturers standard it is necessary to first quantify the α-amylase activity (U/L) within a sample prior to dilution and testing. This can be achieved by using assays for α-amylase activity, such as the Phadebas® Forensic Tube Test (Magle Life Sciences, Lund, Sweden) or the Salimetrics® Salivary α Kinetic Enzyme Assay Kit (Pennsylvania, USA).

Table 2: Reported sensitivity of Phadebas® paper.

Sensitivity (dilution factor)	Reference
1:100	(Breathnach & Moore, 2013; Hedman et al., 2008)
1:250	(Park, Son, Seo, & Lim, 2015)
1:500	(Davidek, 2014)
1:1000	(Roda et al., 2014)

Mullen (2012) has wrongly attempted to assign a value to the lower detection limit of Phadebas® paper by taking the average literature value (350,000 U/L) and applying the dilution factor identified as detectable from one study. By applying this logic to the sensitivity results obtained by Hedman (2008), it was suggested by Mullen that the Phadebas® Forensic Press Test was sensitive to 1750 U/L. However, it is known that there are naturally very large variations of α-amylase activity in saliva samples that exist both within an individual and within the population (discussed in detail in 3.3.1 Intra- and inter- personal α-amylase variations in saliva and other forensically relevant body fluids). Furthermore, the dilution factor that Mullen applied in this logic resulted from an experiment utilising an incubator at 37°C, which is not a step listed in the protocol for the Phadebas® Forensic Press Test. This method was adopted in an attempt to increase sensitivity; it is also known that α-amylase activity is enhanced by temperature (discussed in detail in 3.3.2 Optimal conditions for α-amylase activity: temperature, pH and activators). Thus, it is strongly advised that this information should not be regarded as appropriate reference for conclusions on the sensitivity of Phadebas® paper, nor should this logic be applied to any other results. Interestingly, a study conducted by Olsén et al. (2011), used methods that could have lead to validation of the sensitivity of Phadebas® paper; although this was not within the scope of the study. By using the Phadebas® Forensic Tube Test to quantify the amount of α-amylase activity in the saliva samples, it was demonstrated that a correlation exists between the Phadebas® Forensic Press Test (with respect to the time taken to indicate a positive result) and the α-amylase activity

in a saliva sample. However, unfortunately the detection limits of Phadebas® paper was not explored.

3.2 Reported specificity of Phadebas® paper

As mentioned in the introduction, α-amylase is not specific to either saliva or to humans. Ideally, presumptive screening tests for saliva would be specific for human salivary α-amylase; this is not the case with Phadebas® paper. This emphasises the need for confirmatory testing of saliva following presumptive screening with Phadebas® paper. Two studies (Breathnach & Moore, 2013, 2015) have determined that a rather small proportion (13%) of items that are examined with Phadebas® paper return positive results with the confirmatory test, RSID™-Saliva, which is specific for human salivary α-amylase. This stresses the incidence of false positive reactions and lack of specificity exhibited by Phadebas® paper and the importance of using confirmatory tests for saliva.

There is some confusion in the literature regarding the specificity of Phadebas® paper to the two α-amylase isoforms (salivary and pancreatic) that exist in humans; Mullen (Mullen, 2012) reports that Phadebas® products are specific to only the salivary α-amylase. However, this is the only source that suggests this and it is based on a reference that is no longer available. Hence, it is implied that this information may have been obtained or based on information that was incorrect and as a consequence, it should not be considered as a reliable source of information on this topic. Other literature suggests and proves that this test screens for both pancreatic and salivary amylase (Breathnach & Moore, 2015; Casey & Price, 2010; Magle Life Sciences, 2014; Olsén et al., 2011). The following section outlines the cross-reactivity of Phadebas® paper with other forensically relevant samples, samples of non-human origin and non-biological sources.

3.2.1 Cross-reactivity of Phadebas® paper with other forensically relevant samples

The manufacturer states that many laboratories have determined that no other forensically relevant body fluid will react within 10 minutes following the current protocol, with the exception of faeces (Magle Life Sciences, 2014). However only perspiration, semen and vaginal secretions are considered as the other forensically relevant samples in this specification; all other body fluids samples, including, blood, breast milk, faeces, nasal secretions, tears and urine should be considered. The high level of amylase in fecal matter is due to a combination of both salivary α-amylase and a significant amount of pancreatic α-amylase in the sample, which is indistinguishable from salivary α-amylase by Phadebas® paper (Breathnach & Moore, 2013; Davidek, 2014; Olsén et al., 2011; Watchman et al., 2008) and thus may contain α-amylase activity as high as those found in saliva. However, faecal matter contains visible constituents and can visibly be distinguished from saliva stains. Thus, the cross-reactivity experienced with this body fluid may only be an issue in the incidence of faecal matter on dark fabrics or items heavily stained with blood, soil or other contaminants. Furthermore, Davidek (2014) reported that 90% of neat and dilute saliva stains (n=81) reacted within 20 minutes, with 5% being detected after the 40 minute testing period. So not all saliva stains are detected within the 10-minute time frame, which opposes the manufacturers' specification; this is problematic where specificity is concerned. The manufacturer also claims that generally, α-amylase found in other body fluids will not be present in sufficient quantity for detection using Phadebas® chemistry (Magle Life Sciences, 2014). This statement is based on a study conducted by Whitehead and Kipps (1975), which shows that a factor of 1000 exists between the α-amylase activity present in saliva as opposed to urine (the second highest amylase-containing body fluid, other than faeces). However, many studies since have demonstrated very large and unpredictable inter- and intra- personal variations in α-amylase levels in all body fluids (Auvdel, 1986; Gutowski & Henthorn, 1983; Hedman, Dalin, Rasmusson, & Ansell, 2011; Kipps & Whitehead, 1975) (discussed in detail in 3.3.1 Intra- and inter- personal α-amylase

variations in saliva and other forensically relevant body fluids) and as a result variable observations have been reported in the literature with regards to specificity of Phadebas® paper. Typically, false positive results are not observed with other forensically body fluids. Breathnach and Moore (2013), showed that the Phadebas® Forensic Press Test does not produce positive results within the 40-minute time frame with other forensically relevant body fluids, including blood, urine, semen, vaginal secretions and perspiration, with the exception faecal matter. However, these findings were based on a limited sample size; only two volunteers were used to collect one of each sample, i.e. n=1. Similarly, Watchman et al. (2008) found that only saliva (n=7) and faeces (n=6) produce a positive reaction on Phadebas® paper, when compared to blood (n=8), perspiration (n=5), urine (n=5), semen (n=3) and vaginal secretions (n=2). This study used a larger samples size, however, the number of human sources used to produce these samples was not communicated. Conversely, Olsén et al. (2011) and Davidek (2014) have demonstrated that false positives are possible to encounter with regards to other forensically relevant samples. Olsén et al. (2011) found that other body fluids such as urine (n=10), vaginal fluids (n=10), semen (n=8) and perspiration (n=10) reacted with the Phadebas® paper test under the 10 minute stipulated time frame. Olsén et al. (2011) used an overall larger sample size and demonstrated that samples were collected from different individuals, thereby demonstrating rigour in the scientific experiment. Davidek showed cross-reactivity with urine within the 40-minute reaction time frame; 15 samples from five different donors were evaluated. Overall, there are variable results surrounding the ability of forensically relevant fluids to contain α-amylase activities that overlap with that of saliva and producing false positive results.

3.2.2 Cross-reactivity of Phadebas® paper with non-human biological samples

There is limited research surrounding the cross-reactivity of Phadebas® paper and samples containing amylases including saliva from animals, plant material, and bacterial and fungal

products. Watchman et al. (2008) has described variable reactions of Phadebas® paper with canine and feline saliva. Other animal fluids have not been studied for cross-reactivity with Phadebas® paper; in order to fully comprehend the extent of cross-reactivity of Phadebas® paper, it can be suggested that this is an area for future investigation, especially for domesticated animal body fluid products. The same study also described no cross-reactivity with plant material potentially containing β-amylase, including, wheat, barley, sweet potato and soybean. However, this does not conclusively demonstrate that Phadebas® paper does not produce false positives with substances likely to contain β-amylase (i.e. from plant material, bacteria and fungi) and instead only suggests that α- and β- amylases that may exist in plant material were not present in sufficient quantity to instigate a positive reaction. That being said, β-amylase from such sources is slower to digest starch and may not work within the 40-minute examination period. The potential cross-reactivity of Phadebas® paper with β-amylase (and γ-amylase) has not been investigated. It is apparent that if Phadebas® paper is determined to be an appropriate presumptive screening tool for saliva, cross-reactivity with non-human biological samples likely to contain α-, β-, and γ- amylase need to be thoroughly investigated.

3.2.3 Cross-reactivity of Phadebas® paper with non-biological samples

Aforementioned, amylases are encountered in a number of different industrial processes. Detergent companies often add enzymes, such as α- and β- amylase, to their products as an effective method of breaking down tough stains created by polysaccharides. The possibility that amylases present in common household laundry detergents may contribute to the positive detection of evidentiary α-amylase during screening has been investigated in a single study, pertaining only to the Phadebas® Forensic Tube Test. Feia and Novroski (2013) found that clothing laundered in detergents known to contain the α-amylase enzyme does not retain any detectable levels of amylase following a 'typical' wash cycle. In this experiment, a typical wash cycle referred

to using a warm-cold temperature, light load setting with a light load volume of detergent. This suggests that at some point during the laundering cycle, α-amylase enzymes are damaged, degraded or removed and that detergent enzymes should not contribute to a misidentification of a saliva stain (using the Phadebas® Forensic Tube Test). However, it was found that undiluted detergents deposited onto various fabric swatches resulted in false positive detection of α-amylase activity with the Phadebas® Forensic Tube Test. Since the Phadebas® Forensic Tube Test and the Phadebas® Forensic Press Test function using the same Phadebas® chemistry, it is likely that these findings may apply to both tests, although a study to confirm this inference is advisable. Additionally, the incidence for artificial lubricants such as those found on condoms, or in personal lubricants may be likely to be present when screening for saliva on certain exhibits, especially those relating to sexual assault cases. Similarly, other household items such as cleaning products, condiments, beverages and toiletries may be present on a substrate under examination and may contain α-amylase, thus it is important to understand the cross-reactivity of these with Phadebas® paper. Davidek (2014) undertook experiments related to condoms and personal lubricants and concluded that none of the samples tested (Lifestyles®, Durex®, Trojan®, KY® and NaturePlex™) generated positive results. Additionally, Watchman (2008) investigated the reactivity of Ajax® Spray n' Wipe®, bleach, Pine O Clean®, Coca-Cola® and vinegar, and found no specificity issues concerning Phadebas® paper and these products. It is apparent that these studies are limited and a more comprehensive study should be undertaken to cover all potential amylase-containing products to further determine the suitability of Phadebas® paper as a presumptive screening tool for saliva.

3.3 Factors affecting the sensitivity and specificity of Phadebas® paper

There are many variables that need to be considered during the assessment of sensitivity and

specificity of Phadebas® paper for the presumptive screening of saliva. These include the prevalence of intra- and inter- personal variations of α-amylase activity in saliva and other forensically relevant body fluids; optimal conditions for α-amylase activity; potential effects of the substrates; the occurrence of mixed biological samples; and, the various degradative conditions that saliva may be exposed to in forensic situations. All of these factors (discussed below) relate to the effect of such factors on α-amylase activity, which directly influences the sensitivity and specificity of Phadebas® paper to saliva.

3.3.1 Intra- and inter- personal variations of α-amylase activity in saliva and other body fluids

The amount of salivary α-amylase differs in people of different ethnic groups; the duplication of the α-amylase gene during evolution has led to gene copy number variation. The number of gene copies is directly related to the levels of salivary amylase (Perry et al., 2007). Moreover, α-amylase activity in saliva, and other body fluids, in humans is believed to be significantly affected by factors such as age of the individual (Ben-Aryeh et al., 1986) alcohol consumption (Maruyama et al., 2003), stress (Nater et al., 2006; Nater, Rohleder, Scholtz, Ehlert, & Kirschbaum, 2007), drug use and some illnesses, i.e. pancreatic disorders and in several kind of tumours affecting the digestive apparatus (Kazmierczak, 1997; Sugimoto et al., 2001). The smoking habits of people also clearly reduce the enzymatic activity of salivary α-amylase (Enemchukwu, Ubaoji, Igwilo, & Udedi, 2013; Weiner, Khankin, Levy, & Reznick, 2009). There are a number of studies reporting that both salivary flow rate and saliva composition vary rhythmically over a 24-hour period; α-amylase levels in saliva have shown to be low in the morning and high in the afternoon (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007; Rohleder, Chen, Wolf, & Miller, 2008; Rohleder, Nater, Wolf, Ehlert, & Kirschbaum, 2004). However, there is some conflicting literature that suggests there are no distinct changes in α-amylase in saliva over the course of a day (Yamaguchi et al., 2006). α-Amylase levels have also been investigated with respect to food intake. Results have been variable, however,

patterns show that there are tendencies for amylase activities to be higher after food consumption (Mackie & Pangborn, 1990; Tsutsumi, Higashide, Mizuno, Tamaki, & Katsumata, 1991).

As demonstrated in Table 3, the α-amylase activity that has been reported in saliva from humans is highly variable, with an overall range of 630–3,130,000 U/L amongst a sample size of 197 individuals across only 10 different studies; this is an incredibly small representation of the entire population. It can, therefore, be suggested that the actual range may be greater than this when the entire population is considered. The study by Tsutsumi, Higashide, Mizuno and Katsumata (1991), demonstrated the possibility of the α-amylase activity to fall below the specified lower detection limit of Phadebas® paper (2000 U/L) in dried samples, thus, it is possible that some saliva stains will not have sufficient α-amylase activity to be detected by Phadebas® paper. In fact, there has been

Table 3: Reported activity of α-amylase in human saliva.

Mean (U/L)	Range (U/L)	Number of samples	Sample type	Method of detection	Reference
5,037	630–15,360	52	Dried	Neo Amylase Test	(Tsutsumi et al., 1991)
94,000	5,000–108,000	20	Dried	Phadebas® Amylase Test	(Auvdel, 1986)
240,000	38,000–840,000	10	Fresh	Phadebas® Amylase Test	(Hedman et al., 2011)
291,000	89,000–516,000	8	Dried	Phadebas® Amylase Test	(Gutowski & Henthorn, 1983)
322,000	263,000–376,000	3	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Whitehead & Kipps, 1975)
350,000	72,000–1,300,000	50	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Kipps & Whitehead, 1975)
425,000	185,000–1,018,000	10	Fresh	Phadebas® Amylase Test	(Clem, Maidment, & Ringham, 2001)
2,800,000	1,850,00–3,130,000	10	Dried	Caraway method	(Ohya, Iwasa, Komoriya, Bunai, & Sagisaka, 1986)
-	29,000–500,000	24	Fresh	Phadebas® Amylase Test	(Akutsu, Watanabe, Fujinami, & Sakurada, 2010)
-	≈225,000–≈1,415,000	10	Fresh	Phadebas® Amylase Test	(Olsén et al., 2011)

one published case whereby the α-amylase activity in a fresh saliva sample was unable to be detected (Bitner, Clark, Priestley, & Ziencik, 2013) (using the Phadebas® Forensic Tube Test). The incidence of this situation is not known in the population and may affect the usefulness of α-amylase as the molecular target for the presumptive testing and screening for saliva.

Some of the variation observed may be attributable to the different methods utilised for the determination of α-amylase activity across the conducted studies. The range exhibited with the use of the Phadebas® Amylase Test (using the same chemistry as Phadebas® paper), is 5,000–≈1,415,000 U/L. Furthermore, the dried saliva stains overall displayed less α-amylase activity (5,000–516,000 U/L) than the fresh saliva samples (29,000–≈1,415,000 U/L), which is in line with observations concerning the drying or ageing of samples and the decrease in α-amylase activity (discussed in detail in 3.3.3 Drying of saliva and the age of the saliva stain).

The incidence of α-amylase variation in other forensically relevant human body fluids is outlined in Table 4. Only faeces, semen, tears and urine in the following studies display levels greater than the specified lower detection limit of Phadebas® paper (2000 U/L). However, as mentioned for the reported activity of α-amylase in human saliva, this is only a small representation of the entire population and the actual range may be greater than this. Furthermore, many of these studies have identified the α-amylase activity in fresh samples, i.e. the sample has not been exposed to drying or ageing, which has been shown to decrease α-amylase activity (discussed in detail in 3.3.3 Drying of saliva and the age of the saliva stain). Overall, the incidence of α-amylase activity in other forensically relevant samples (other than faeces) appears to be several orders of magnitude lower than those found in human saliva; it is entirely possible that specificity of Phadebas® paper to saliva may be maintained through this phenomenon, with the exception of faeces.

Table 4: Reported activity of α-amylase in other forensically relevant body fluids.

Body fluid type	Mean (U/L)	Range (U/L)	Number of samples	Sample type	Method of detection	Reference
Blood (serum)	160	84–300	49	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Kipps & Whitehead, 1975)
Blood (whole)	-	110	1	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Whitehead & Kipps, 1975)
	-	510–630	5	Fresh	Phadebas® Amylase Test	(Akutsu et al., 2010)
Breast milk	576	425–810	4	Dried	Neo Amylase Test	(Tsutsumi et al., 1991)
Faeces	-	≈10,000–≈500,000	6	Dried	Phadebas® Amylase Test	(Willott, 1974)
Nasal secretions	232	56–536	8	Dried	Neo Amylase Test	(Tsutsumi et al., 1991)
Perspiration	575	45–895	4	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Kipps & Whitehead, 1975)
	-	90–395	5	Fresh	Phadebas® Amylase Test	(Akutsu et al., 2010)
Semen	95	28 –200	3	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Kipps & Whitehead, 1975)
	104	7 –1,620	111	Dried	Phadebas® Amylase Test	(Auvdel, 1986)
	-	41	1	Dried	Phadebas® Amylase Test	(Gutowski & Henthorn, 1983)
	-	35	1	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Whitehead & Kipps, 1975)
	-	2,100	1	Fresh	Phadebas® Amylase Test	(Akutsu et al., 2010)
Semen (vasectomised)	48	1–345	32	Dried	Phadebas® Amylase Test	(Auvdel, 1986)
	1725*	20 –6,820	4	Dried	Phadebas® Amylase Test	(Auvdel, 1986)
Semen (aspermic)	-	18	1	Dried	Phadebas® Amylase Test	(Auvdel, 1986)
Tears	-	870–2,150	2	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Kipps & Whitehead, 1975)
Urine	599	263–940	3	Fresh	Plate diffusion (Procion® Red MX-2B-amylocpectin)	(Whitehead & Kipps, 1975)
	850	130–3,500	18	Fresh	Plate diffusion (Procion® Red MX-	(Kipps & Whitehead,

					2B-amylocpectin)	1975)
	-	228–1,080	9	Dried	Phadebas® Amylase Test	(Gutowski & Henthorn, 1983)
	-	480–595	5	Fresh	Phadebas® Amylase Test	(Akutsu et al., 2010)
Vaginal secretions	51	45–60	3	Dried	Neo Amylase Test	(Tsutsumi et al., 1991)
*Excluding the sample with amylase activity of 6820 U/L the average remaining samples would be 27 U/L.						

3.3.2 Optimal conditions for α-amylase activity: temperature, pH and activators

As for all enzymes, salivary α-amylase has optimal conditions for maximum activity. These conditions (in vitro) are a temperature of 37°C (i.e. body temperature) and a pH of 7.0 (Rudeekulthamrong & Kaulpiboon, 2012). Furthermore, salivary α-amylase is a metalloenzyme and has an absolute requirement for calcium as a cofactor to function (in addition to chloride ions). However, according to the manufacturers' instructions (Magle Life Sciences), tests are undertaken at room temperature and the item under examination and paper are kept damp with distilled water (pH ≈7.0). The manufacturer reports that the Phadebas® Forensic Press Test is less sensitive to that of the Phadebas® Forensic Tube Test when both are being used for qualitative analysis. The reasoning for this may lie within the fact that the Phadebas® Forensic Tube Test involves incubation at 37°C i.e. the optimum enzyme temperature for α-amylase activity. Perhaps protocol for Phadebas® paper also needs to be revised to incorporate the optimal conditions, especially temperature, for α-amylase activity to potentially increase the sensitivity of this test.

A study by Hedman et al. (2008) demonstrated that the use of an incubator (at 37°C) with Phadebas® paper, resulted in a higher sensitivity of Phadebas® paper to the sample; a dilution of 1:200 could be detected at 37°C, as opposed to 1:100 at room temperature. However, these results were highly variable and could not be accurately replicated in the same experiment.

Additionally, Forensic Science South Australia (FSSA) has developed protocol for Phadebas® paper that differs from the Phadebas® Forensic Press Test procedure, specified by the manufacturer. FSSAs current protocol involves incubation at 37°C for 45 minutes, especially when low activity of α-amylase is likely to be present (Skrinnikoff, Donnelly, & Silenieks, 2016). Furthermore, they have recommended extending the presumptive test time to 90 minutes at room temperature when incubation is not possible (Skrinnikoff et al., 2016) since it takes this amount of time to produce similar observations at the two temperatures.

The effects of pH have yet to be investigated with respect to increasing sensitivity of Phadebas® paper. Adjusting the pH of the Phadebas® paper/forensic exhibit wetting agent is achievable with a water-soluble calcium salt, such as calcium chloride ($CaCl_2$). This salt is theoretically an ideal substance to use for this purpose since the dissociated ions in solution both lower the pH and are required for α-amylase activity. However, this is inherently more difficult to achieve since there may be undesirable interactions with such pH-adjusting substances and the saliva/body fluid specimen, and may also affect the downstream processing of DNA analysis; temperature is much easier to manipulate and the outcomes on downstream processing such as DNA analysis are much more predictable. That being said, it is known that distilled water can rupture the cell wall due to the fact that is it hypotonic to the cell membrane (Hardin, Bertoni, Kleinsmith, & Becker, 2012), thus epithelial cells present in the saliva stain may be damaged as a result of this. Alternatively, an isotonic saline solution (pH ≈5.5) is only slightly outside the optimal pH range for α-amylase activity and could be used instead of distilled water to potentially preserve DNA evidence; perhaps even a calcium salt pH-adjusted solution could overcome this issue (discussed further in 4.3.2 Phadebas® paper and the recovery of DNA evidence).

It can be suggested that increasing the sensitivity of the Phadebas® paper may consequently

reduce specificity by allowing other forensically relevant samples to react. However, results from Watchman et al. (2008) suggest otherwise. This experiment involved incubating Phadebas® paper and samples under examination at 37°C for 45 minutes (as per FSSA protocol) and found that no false positive reactions were produced with any other forensically relevant samples (except faeces). This study provides a basis for which protocol changes can be suggested; by increasing sensitivity without sacrificing specificity, the effectiveness of the Phadebas® Forensic Press Test can be enhanced.

3.3.3 Drying of saliva and age of the saliva stain

A study undertaken by Tsutsumi et al. (1991) identified that the α-Amylase activity in saliva stains drops rapidly during the first hour of drying (to approximately 25% of the initial α-amylase activity), and continues decreasing to approximately 17% of initial α-amylase activity in the first 24 hours, at room temperature (20°C). Continued drying at room temperature over the course of one month found that the α-amylase activity dropped to approximately 3% of initial α-amylase activity. Similarly, stains kept at -20°C and -80°C exhibited a similar substantial decrease in α-amylase activity in the first 24 hours, due to drying, however, the α-amylase activity within the stains following this initial drop remained practically constant over the next 28 days when stored at these low temperatures. Comparably, Miwa (1982) investigated the variability of α-amylase activity in saliva stains kept at room temperature (15-20°C), 37°C and 5°C for one month and described that the stains kept at 5°C showed little decrease in α-amylase activity, while the stains kept at room temperature and 37°C showed remarkable decrease. These studies highlight the limitations of Phadebas® paper with respect to aged stains. Since saliva stains encountered in forensic investigations are typically exposed to uncontrolled temperatures and therefore decreases in α-amylase activity are likely to follow the pattern described for room temperature in Tsutsumi et al. (1991); the Perth metropolitan area, on average, experiences temperatures between 12.8–24.7°C

(Bureau of Meteorology, 2017). It is inferred that Phadebas® paper may not be capable of detecting saliva stains that are greater than 28 days old based on the study by Tsutsumi (1991). Yet, Watchman et al. (2008) have described a positive indication for α-amylase activity in saliva stains up to two years old. However, the storage conditions of these samples were not disclosed, which may have played a part in preserving the α-amylase activity in the samples. Furthermore, Gutowski and Henthorn (1983) demonstrated that after 49 days of storage in undefined conditions saliva stains only exhibit 1% of their original α-amylase activity; this was much quicker than the decrease in α-amylase activity found in Nelson and Kirk (1963). However, observations relating to the effects of drying/age of the sample depend entirely on the starting α-amylase activity within the saliva specimen and the subsequent environmental conditions that the saliva is exposed to in a forensic scenario. Based on this logic it is possible that that results from aged saliva stains and fresh stains originating from another α-amylase containing body fluid or source could be similar, i.e. the sensitivity and specificity of Phadebas® paper may be compromised as a result of limited knowledge of the age of the potential saliva sample.

3.3.4 Mixed body fluid stains

When considering saliva in a forensic context (especially sexual assault cases), it is not unusual to encounter saliva in the presence of other forensically relevant fluids, especially blood, semen, urine and/or vaginal secretions. Thus, it is important to consider the sensitivity and specificity of Phadebas® paper with saliva when it exists as a mixed sample. Limited knowledge exists regarding the detection of saliva in mixed biological samples. However, the common theme within the available literature suggests that saliva can still be accurately detected in the presence of a contaminating substance such as blood, semen, urine or vaginal secretions.

Whole blood and vaginal secretions have been shown in one particular study to decrease α-

amylase activity in saliva, this is thought to be due to the high blood protein content (Tsutsumi et al., 1991), suggesting that if a stain containing saliva and blood or vaginal secretions was encountered then a false negative could result with Phadebas® paper. Whole blood and vaginal secretions were the only body fluids used to investigate the effects of mixtures on the activity of α-amylase in saliva; urine and semen, nor any other forensically relevant body fluid were examined in this study. Despite this, research since has demonstrated that the existence of blood in a saliva sample does not interfere with the detection of saliva using Phadebas® paper (Breathnach & Moore, 2013; Vandenberg & van Oorshot, 2006). Breathnach and Moore (2013) have described that reactions of Phadebas® paper with 1:10 dilutions of saliva with whole blood, urine and semen are capable of producing a positive indication for α-amylase activity. Therefore it was concluded that the substances present in blood, urine and semen do not inhibit the enzymatic activity of α-amylase or the starch polymer-dye complexes within the Phadebas® paper. Similar conclusions were drawn from a study by Roda et al. (2014), whereby multiple ratios of mixtures of saliva in blood and semen produced detectable observations with Phadebas® paper.

Phadebas® paper has been used in an attempt to demonstrate the detection of saliva to identify expectorated blood spatter (Park et al., 2015). In this particular study, the presence of blood was limited by the ability of the investigator to observe the results of Phadebas® paper, since the intense red colour of the blood interfered with the visibility of the blue colour change. This effect was lessened where lower concentrations of blood in saliva was concerned. However, this issue may be associated with the improper use of Phadebas® paper in this experiment; the saliva and blood mixture was directly deposited onto the Phadebas® paper whereas in practice dried saliva and blood mixtures are more likely to be encountered. Thus, the intensity of the red coloration exhibited by the blood in this method may not transfer to the Phadebas® paper when dried samples are examined, and consequently, the colour interference may not be as problematic in

practice. In fact, observations from Davidek (2014) showed that the transfer of blood from a fabric swatch onto Phadebas® paper is less severe when compared to observations in Park et al (2015).

Overall, the detection of saliva in mixed human biological samples with Phadebas® paper does not appear to be inhibited by the presence of an accompanying body fluid. It can be suggested that in some instances the presence of a body fluid mixture may enhance the detection of saliva since all body fluids contain some α-amylase activity. However, further studies need to thoroughly investigate the detectability of saliva in mixed biological samples, especially vaginal secretions.

3.3.5 Influence of the substrate

The potential influence of the substrate with respect to the use of Phadebas® paper has not been sufficiently addressed. It may be that certain dyes or chemicals within fabrics diminish the activity of the α-amylase enzyme and therefore its detectability with Phadebas® paper, as is the case with indigo dye in denim on the Taq polymerase enzyme, used in polymerase chain reaction (PCR) for DNA amplification (Goodwin, Linacre, & Hadi, 2011); this is yet to be suggested in the literature and has not been researched. Many of the sensitivity and specificity studies conducted for Phadebas® paper deposit saliva onto cotton substrates without knowing the implications of doing so (Breathnach & Moore, 2013; Davidek, 2014; Hedman et al., 2008; Olsén et al., 2011; Roda et al., 2014; Watchman et al., 2008). Additionally, the porosity of the substrate may also contribute to the sensitivity of the Phadebas® paper with respect to the transference of the saliva stain to the paper. It can be suggested that more porous substrates will retain the saliva stain and transference of material to the Phadebas® paper will be minimal. Conversely, on non-porous substrates, the ability of the Phadebas® paper to interact with a saliva stain will be increased, resulting in an improved transfer of the stain to the Phadebas® paper, subsequently increasing detectability of the saliva stain. In fact, Watchman et al. (2008) has suggested that material effects should be

considered since it was established that thinner materials may give weaker reactions. A more robust method for the identification of a potential correlation between the detectability of a saliva stain and the porosity of the substrate needs to be implemented. This can be achieved simply by determining the porosity of a substrate (using the saturation method and equation (1), below) and graphing this against the strength of the reaction with Phadebas® paper.

Calculating porosity experimentally by saturation:

In this method, the volume of the substrate (V_t) is determined and placed in a known volume of water. The displacement of the water $(\Delta Volume_{H2O})$ is then observed and substituted into equation (1) along with the volume of the substrate (V_t).

$$Porosity\ (P_t)\ \% = \frac{Pore\ volume\ (V_p)}{Total\ volume\ (V_t)} \times 100, \quad (1)$$

where, V_t is equal to the $L \times W \times H$ of the substrate, and V_p is equal to $V_t - \Delta Volume_{H2O}$.

3.3.6 Denaturation of α-amylase

The exposure of saliva and resulting stains to a number of external factors such as sunlight (UV light), variable ambient and storage temperatures, humidity, and chemical agents, is often inevitable in a forensic scenario, each of which may have adverse effects on the activity of α-amylase. Of particular concern is the denaturation of α-amylase; if α-amylase activity cannot be detected in a sample as a result of enzyme degradation, Phadebas® paper will be of little use for the presumptive screening of saliva.

It is apparent that storage at room temperature will result in a more rapid decline of α-amylase activity, suspected to be as a result of enzyme denaturation (as discussed in 3.3.3 Drying of saliva and the age of the saliva stain). Therefore it will be suggested that forensic exhibits required to

undergo presumptive screening for saliva with Phadebas® paper should be kept in a fridge or freezer until the examination can be conducted, in order to maximise α-amylase activity retention. However, like all enzymes, repeated thawing and freezing may be detrimental to the α-amylase enzyme, therefore its activity (Cao, Chen, Cui, & Foster, 2003), and should be avoided.

The effects of UV light on α-amylase activity have been explored by Roda et al. (2014), whereby a saliva stain exposed to UV light (in a UV crosslinker) exhibited a delayed positive indication for α-amylase activity on Phadebas® paper when compared to a neat saliva sample. This suggests that α-amylase activity was impacted, i.e. the enzyme was denatured or degraded. Thus, it is possible that the use of UV ALS for the screening of saliva on forensic exhibits may contribute to the degradation of the α-amylase enzyme. Furthermore, a saliva stain exposed to both high temperatures and UV light (in a car window) showed a longer delayed reaction with Phadebas® paper. Thus, it is important to consider both the type of forensic examination required on exhibits at the crime scene for appropriate storage, in addition to the potential effects of the presumptive and screening techniques used in the forensic laboratory.

It is also entirely possible that saliva on forensic exhibits will be exposed to chemical cleaning agents, such as bleach, detergents, soaps, etc., the effects of which have scarcely been considered with respect to α-amylase activity. Sodium dodecyl sulphate (SDS) is an anionic surfactant found in many cleaning and hygiene products and has been shown to cause the destabilisation of the α-amylase enzyme (Shareghi & Arabi, 2008). Thus, it is proposed that the use of Phadebas® paper on forensic exhibits containing saliva, following an attempted clean up will be ineffective.

4 Integrated forensic approach for exhibits containing saliva stains and other evidence

The development of an integrated forensic approach is necessary to maximise evidence recovery and to ensure that a particular treatment is not detrimental to other types of evidence that may be present on a single exhibit. The concept of an integrated forensic examination is gaining popularity in modern forensics for this reason. However, there are limited recommendations and guidelines for such methodology, especially pertaining to Phadebas® paper and saliva.

4.1 Phadebas® paper and the detection of semen

In cases where both semen and saliva are expected to be present, the manufacturer suggests that Phadebas® paper can be used in conjunction with the acid phosphatase (AP) test for semen. Once presumptive screening with Phadebas® paper for saliva has been established, AP can be used directly onto the paper. This is may also additionally be useful if semen stains produced a false positive reaction with Phadebas® paper. However, there have been no studies conducted to validate the effectiveness of this technique.

4.2 Phadebas® paper and the recovery of DNA evidence

The ultimate goal, subsequent to identifying any biological evidence, including saliva, is to generate a DNA profile. Hence, it is important to understand the implications that any forensic examinations undertaken on an exhibit may have on the downstream processing of such samples; for instance, Phadebas® paper may introduce inhibitors to PCR amplification. Roda et al. (2014) has tested the possible implications of DNA analysis following the Phadebas® Forensic Press Test by collecting

samples from both the substrate and the Phadebas® paper and subjecting them to DNA analysis. It was determined that the use of Phadebas® paper did not inhibit DNA analysis, however, samples collected from the fabric are necessary to recover full profiles. This conclusion is supported by Davidek (2014), who found that obtaining profiles from Phadebas® paper following DNA analysis is not as effective as excising the sample directly from the item or exhibit under examination. Roda et al (2014) also reported that Phadebas® paper does not detect all possible stains that contain sufficient DNA to produce a DNA profile, however, this conclusion was only based on observations from one sample. However, while the identification of α-amylase activity can be indicative of human saliva, the activity of α-amylase is not proportionate to the amount of DNA in the stain (Hedman et al., 2011). This can be explained since α-amylase is an extracellular enzyme produced in the salivary glands, and the amount of DNA in saliva depends on the shedding of buccal cells in the oral cavity. Hence, the detection of saliva with Phadebas® paper does not guarantee the generation of a DNA profile, and vice versa.

It was suggested in section 3.2.2 Optimal conditions for α-amylase activity: temperature, pH and activators, that the use of pH neutral water in the Phadebas® Forensic Press Test may damage epithelial cells contained in saliva stains and thereby compromise the quality of the DNA since it is hypotonic to the cell membrane. This is an important concept to study since a substantial amount of water is used in the test to keep both the exhibit and Phadebas® paper damp, however, no studies to date have identified this as a potential issue.

5 Proposed methodology for the validation of Phadebas® paper as a presumptive screening tool for saliva on forensic exhibits

This section identifies crucial areas that need to be investigated for the implementation of Phadebas® paper into the PathWest forensic laboratory and outlines the methodology that will be adopted for the validation of the Phadebas® Forensic Press Test. An attempt to negate the issues highlighted in the review will be made; scientific rigour is essential in the experiment to follow, as this lacks in many of the previous studies addressed. Finally, recommendations for future studies of the concepts outlined in this review will be suggested.

5.1 Sensitivity study

It is clear from this review that no studies have attempted to confirm the lower detection limit of Phadebas® paper, as specified by the manufacturer (2000 U/L). In order to accomplish this, a quantitative method (i.e. the Phadebas® Forensic Tube Test) will be applied to determine the starting α-amylase activity in a single saliva sample. This sample will subsequently be used to create dilutions of saliva (to be determined once the starting α-amylase activity is confirmed). Neat saliva and distilled water will be used as the positive and negative controls, respectively. The Phadebas® Forensic Press Test will be carried out as per a modified version of the manufacturers' instructions (Magle Life Sciences). This will involve depositing a wet sample onto the Phadebas® paper in order to negate the effects of drying of the sample on α-amylase activity. It is acknowledged that this does not replicate a forensic situation whereby saliva stains are encountered (rather than fresh/liquid saliva), however, the aim of this study is to validate the

Phadebas® paper, which does not require such circumstances to be mimicked. Furthermore, if the samples were permitted to dry, the results would not be interpretable, or the effects of drying would have to be investigated to produce a factor that could be applied to the resulting α-amylase activity of the samples; the proposed method is far less cumbersome. Secondly, the effects of temperature on the sensitivity of Phadebas® paper to saliva is to be investigated, which may allow the introduction of a revised protocol in the use of the Phadebas® Forensic Press Test. The samples described above will be used also in this part of the study. However, instead of undertaking the examination at room temperature, it will be carried out in an incubator at 37°C.

5.2 Specificity study

Somewhat variable results have been obtained with the reported specificities of Phadebas® paper to other forensically relevant body fluids which challenge the specifications claimed by the manufacturer (that no other forensically relevant body fluid will react within 10 minutes following the current protocol, with the exception of faeces). For the purpose of this study, other forensically relevant body fluids will be defined as blood, breast milk, faeces, nasal secretions, perspiration, saliva, semen, tears, urine and vaginal secretions. The incidence of intra- and inter- personal variations in α-amylase activity will be encouraged in this study in order to maximise the variation experienced in the population. As a result, age, gender, time of the day the samples is taken, etc., will not be controlled in the collection of samples from human participants. The various samples collected will be deposited onto glass and left to dry for a specified time (24 hours) before examination with Phadebas® paper. Again, it is acknowledged that this does not replicate a forensic situation whereby saliva stains are encountered in terms of substrate, however, the choice of a non-porous substrate will allow for maximum transfer between the substrate/stain and the Phadebas® paper and to minimise substrate effects (with respect to porosity). The drying of the sample is to replicate a forensic situation whereby exhibits are likely to contain biological fluid

stains more than a day old. The Phadebas® Forensic Press Test will be carried out as per the manufacturers' instructions (Magle Life Sciences), with neat saliva and distilled water as the positive and negative controls, respectively. The effects of temperature on the specificity of Phadebas® paper, where other forensically relevant body fluids stains are concerned, will also be investigated. The samples described above will also be used for this part of the study, however, will be examined in an incubator at 37°C.

5.3 Detection of saliva in mixed body fluid stains

Aforementioned, it is not unusual for saliva to be present on substrates with an accompanying forensically relevant body fluid. In this study, the other forensically relevant body fluids are defined as blood, semen, urine and vaginal secretions. This choice of fluids pertains to the possibility of these fluids to become mixed with saliva in sexual assault cases. Faeces will not be investigated here due to the fact that samples originating from this source are known to produce strong positive indications for α-amylase activity, thus saliva is not distinguishable in such mixtures; this will be demonstrated in the specificity study. Samples of saliva:blood, saliva:semen, saliva:urine and saliva:vaginal secretions will be mixed in varying ratios (1:0, 3:1, 1:1, 1:3, 0:1). These mixtures will also be deposited on glass and left to dry for 24 hours for the previously mentioned reasons. The drying should additionally lessen the intensity of the red colouration from the blood, thereby minimising the interference with observational interpretation. The Phadebas® Forensic Press Test will be carried out as per the manufacturers' instructions (Magle Life Sciences), with neat saliva and distilled water as the positive and negative controls, respectively.

5.4 Influence of the substrate

It has been highlighted in this review that little consideration of the potential influence of the

substrate has been acknowledged, especially pertaining to the sensitivity of Phadebas® paper to saliva. This study will thereby investigate potential substrate porosity effects on the detectability of saliva with Phadebas® paper. A saliva sample known to elicit a moderately strong positive result (from the sensitivity study) will be deposited onto a number of different substrates, including, textiles (cotton, leather, denim, wool, polyester, nylon, rayon, lycra, flannelette, velvet, lace, etc.), and common household substrates (glass bottle, mirror, aluminium can, paper, glossy, paper, tissue, plastic bag, brick, tile, linoleum, carpet, wood laminate, etc.). The reason for using a saliva sample of known α-amylase activity (known to produce a moderately strong positive result on Phadebas® paper) is so that the potential interference is more apparent; a saliva sample that produces strong positive results will be less likely to allow inhibitory effects to be observed. The Phadebas® Forensic Press Test will be carried out as per the manufacturers' instructions (Magle Life Sciences), with neat saliva and distilled water as the positive and negative controls, respectively. The potential correlation between porosity of the substrate and sensitivity of Phadebas® paper, the porosity of the substrate will be determined, using equation (1), and plotted against the observed result on Phadebas® paper (including the intensity of the reaction and time taken to achieve the result). Substrates that do not follow the trend, may infer possible chemical/dye interferences of the substrate.

5.5 Sample storage and handling

As per section 3.3.3 Drying of saliva and age of saliva stains and 3.3.6 Denaturation of α-amylase, body fluid samples will be stored in a freezer (≈-4°C) upon collection to maximise the retention of α-amylase activity; furthermore, repeat thawing/freezing of samples will not be practised in order to preserve α-amylase activity.

6 Recommendations for future studies

There are a number of concepts explored in this review that are not being explored in the study outlined above. The reason for this is that the elements considered in the proposed experiment are immediately required for implementation of Phadebas® paper into the PathWest forensic laboratory; the other areas discussed in this review are useful to further strengthen and understand the use of Phadebas® paper, but are not necessary for its validation as a presumptive screening tool for saliva. Given that the proposed experiment has the desired outcome (i.e. the research shows that the paper is suitable for its suggested use), then further testing should commence on the following areas.

A comparison of the screening of saliva with ALS should be compared to Phadebas® paper such that the relative effectiveness for the cost of the method can be identified. Cross-reactivity of Phadebas® paper with non-human biological, and non-biological, samples needs to be investigated if Phadebas® paper is determined to be the most efficient screening tool for saliva. Adjustment of the pH for the wetting agent for dampening Phadebas® paper and the substrate (currently distilled water) can be explored; calcium salts ($CaCl_2$) and isotonic saline solution have been suggested for this purpose, which could further be integrated into a study on the effects of Phadebas® paper protocol and DNA evidence recovery. Specific methods for integrated forensic approaches should be verified, especially for the detection of semen and the recovery of DNA evidence, following Phadebas® paper testing for saliva. The limitations of Phadebas® paper with respect to the detection of aged saliva stains should be assessed; this will not be undertaken in the proposed experiment due to time restrictions. Finally, the extent of the denaturation of the α-amylase enzyme when exposed to external factors, such as sunlight, inconstant ambient temperatures,

humidity and chemical agents should be explored in order to establish the persistence of the enzyme as it exists in forensic situations.

7 Summary of the literature and research aims

The successful detection and identification of saliva stains on exhibits, especially pertaining to volume crime and sexual assault cases is crucial in the development of criminal investigation. However, the detection of salvia is inherently difficult, since it contains no readily visible constituents; hence, screening tools need to be utilised. Currently, PathWest applies ALS technology to screen for saliva; however, this method has been known to fail to detect up to 40% of saliva stains on exhibits. Thus, Phadebas® paper has been suggested as an alternative screening tool for implementation at PathWest, with the added benefit of possessing qualities as a presumptive test. Phadebas® paper is based on the detection of the activity of the enzyme, α-amylase, which is present in very high levels in saliva. This particular presumptive screening test is one-of-a-kind, in that is the only pre-manufactured enzymatic presumptive screening tool that is able to screen for α-amylase activity while simultaneously localising stains. Being pre-manufactured is highly desirable since quality assurance and test standardisation is managed by the manufacturer. However, despite the apparent popularity of Phadebas® paper, this review has highlighted the limits of it as a presumptive screening tool and has demonstrated that attempts at validation lack scientific rigour; this can have significant implications in the interpretation and inclusion of test results into forensic investigations.

In summary, this review has demonstrated the potential for Phadebas® paper to be an effective presumptive screening tool for saliva on forensic exhibits. It has additionally identified a number of areas that need consolidation before Phadebas® paper is implemented into the PathWest forensic laboratory for this purpose. As demonstrated by this review, five crucial areas have been identified as the necessary topics to be addressed in order to determine the suitability of Phadebas® paper as a presumptive screening tool for saliva on forensic exhibits, namely, sensitivity, specificity,

detection of saliva in mixed body fluid stains, influence of the substrate, and protocol recommendations. Thus, the aims of the proposed study are as follows: (1) to validate the lower detection limit of α-amylase activity with Phadebas® paper; (2) to validate the cross-reactivity of Phadebas® paper with other forensically relevant samples, including, blood, breast milk, faeces, nasal secretions, perspiration, semen, tears, urine and vaginal secretions; (3) to investigate the ability of Phadebas® paper to detect saliva in mixed body fluid stains, i.e. saliva:blood, saliva: semen, saliva:urine and saliva:vaginal secretions; (4) to assess the influence of the porosity of the substrate on the detection of saliva with Phadebas® paper; and, (5) to evaluate the effects of examination temperature on the sensitivity and specificity of Phadebas® paper to potentially introduce modifications to the protocol of the Phadebas® Forensic Press Test.

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PART 2

Manuscript

The evaluation and validation of Phadebas® paper as a presumptive screening tool for saliva on forensic exhibits

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Abstract

Phadebas® paper is utilised in the Phadebas® Forensic Press Test for the detection of saliva. The assessment of Phadebas® paper for this purpose has not been studied extensively. The suitability of Phadebas® paper as a presumptive screening tool for saliva on forensic exhibits, was investigated by analysing the following: (1) sensitivity, (2) specificity, (3) effects of temperature on sensitivity and specificity, (4) detection of saliva in saliva:body fluid mixtures, (5) influence of substrate porosity. The results of this study demonstrated that Phadebas® paper is more sensitive to α-amylase activity and less specific for saliva than previously reported. The use of an examination temperature of 37°C has no effect on sensitivity, but increases the incidence of cross-reactivity with other forensically relevant body fluid stains. Blood, urine and vaginal secretions can inhibit the detection of α-amylase activity with Phadebas® paper in mixed saliva:body fluid stains. Substrate porosity is a weak predictor for the time taken for a saliva stain to achieve a strong positive result on Phadebas® paper. Overall, this study has demonstrated that the Phadebas® Forensic Press Test has limitations as a presumptive test for the accurate identification of saliva.

Keywords: Phadebas® paper, saliva, sensitivity, specificity, mixed sample interference, substrate porosity influence

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1 Introduction

Biological evidence originating from saliva can be of considerable importance in criminal investigations; the detection and identification of saliva at a crime scene or on an exhibit is crucial in establishing physical presence, or interaction, as well as inferring actions within a scene (Maloney & Housman, 2014). The forensic significance of saliva is often understated, yet the primary source of deoxyribonucleic acid (DNA) evidence recovered from volume crime scenes as reported in the United Kingdom arises from saliva, typically located on peripheral items such as drinking vessels and cigarette ends (Bond & Hammond, 2008). Furthermore, establishing the nature of, and involvement in, a physical or sexual assault may rely on the detection of saliva on exhibits such as swabs, clothing or bedding from actions such as biting, saliva expectoration or oral intercourse (Breathnach & Moore, 2013, 2015). Regardless of the means by which saliva becomes deposited at crime scenes or on exhibits, the practising forensic scientist is faced with one fundamental issue; the detection (or more specifically the localisation) of saliva.

Saliva stains lack readily visible constituents and are therefore difficult to observe with the naked eye (Gunn, 2009; Humphrey & Williamson, 2001). Consequently, screening techniques such as alternate light sources (ALS) or enzymatic screening techniques are required to enhance visualisation and detection. A 450 nm light source viewed through an orange filter is typically considered the most useful ALS method for observing saliva (Vandenberg & van Oorshot, 2006). However, it is difficult to distinguish between stains originating from saliva and those from other body fluids, especially semen (Gunn, 2009; Vandenberg & van Oorshot, 2006), and the fluorescence exhibited is typically weaker than for other body fluids (Fiedler et al., 2008; Jones, 2005; Miranda, Prado, Delwing, & Júnior, 2014). For these reasons there is a requirement for a more sensitive and specific technique for the localisation and identification of saliva.

A number of enzymatic screening techniques, including Phadebas® paper (Magle Life Sciences, Lund, Sweden), have been developed for this purpose. The preferred molecular target for the enzymatic detection of saliva is α-amylase (EC 3.2.1.1), due to its persistence and abundance in saliva stains (Nelson & Kirk, 1963). In human saliva, α-amylase catalyses the random hydrolysis of α-1,4-glucosidic linkages in dietary starch, glycogen, and related poly- and oligo- saccharides (Gene [Internet], 2004). Phadebas® chemistry exploits this activity of α-amylase: water-insoluble starch polymers are covalently bound to blue dye (CAS RN® 163062-28-0) molecules, which become liberated in the presence of α-amylase. For Phadebas® paper, these starch-dye complexes are immobilised onto filter paper and visualisation of α-amylase positive areas on exhibits occurs when the blue dye molecules diffuse through the pores of the filter paper. The major issue concerning the use of Phadebas® paper for the localisation of saliva is that α-amylase may also be present in other forensically relevant body fluids (Akutsu, Watanabe, Fujinami, & Sakurada, 2010; Auvdel, 1986; Gutowski & Henthorn, 1983; Kipps & Whitehead, 1975; Tsutsumi, Higashide, Mizuno, Tamaki, & Katsumata, 1991; Willott, 1974).

Studies on the effectiveness of Phadebas® paper as a presumptive screening tool for saliva are limited and require additional evaluation (Breathnach & Moore, 2013; Davidek, Unpublished results; Feia & Novroski, 2013; Hedman, Gustavsson, & Ansell, 2008; Olsén, Edenberger, Mattsson, & Ansell, 2011; Park, Son, Seo, & Lim, 2015; Roda, Lee, Barloewen, & Mehmet, 2014). Detailed knowledge of the limitations of Phadebas® paper is crucial for the correct interpretation and inclusion of test results into forensic investigations. Therefore, this study aims to assess the suitability of Phadebas® paper as a presumptive screening tool for saliva on forensic exhibits. In order to achieve this, five areas have been identified for validation. Specifically, the sensitivity and specificity, and effect of temperature on the sensitivity and specificity of Phadebas® paper will be evaluated. Additionally, the ability of Phadebas® paper to detect α-amylase activity in mixed

saliva:body fluid stains will be examined. Finally, the influence of substrate porosity on the ability of Phadebas® paper to detect saliva will be analysed.

2 Materials and methods

2.1 Sample collection, storage and handling

After approval from the Human Research Ethics Committee (HREC) of Murdoch University (2017/040), human saliva (whole), blood (venous), faeces, nasal secretions, perspiration, semen, tear fluid, urine, and vaginal secretions were obtained from a total of five donors (two females and three males) aged between 22 and 60 years. Not all donors submitted each body fluid type for analysis and some submitted multiple samples of the same body fluid. To maximise variation in α-amylase activity, donors were instructed to provide samples at different times of the day. Saliva, perspiration, semen and urine were collected in sterile specimen containers, and faecal, nasal, tear and vaginal samples were collected using sterile rayon swabs. All samples (with the exception of blood) were immediately stored at -10°C to preserve α-amylase activity (Miwa, 1982; Tsutsumi et al., 1991); blood was used immediately upon extraction. When required for use, frozen samples were thawed at room temperature (23°C) and liquid samples were vortexed briefly. All frozen samples were used only once to avoid repeat freeze-thaw cycles.

2.2 Determination of sensitivity

2.2.1 Quantification of α-amylase activity

A stock solution of neat saliva was collected from a single donor and was separated into four specimen containers. One sample was used for the quantification of α-amylase activity in the stock saliva solution. Quantification was achieved (in triplicate) using the Phadebas® Amylase Test (Magle Life Sciences, Lund, Sweden) following the manufacturers' procedure for high α-amylase activities

(Magle Life Sciences, 2007). The absorbance of the supernatant was measured at 620nm using a Hitachi U-1100 spectrophotometer.

2.2.2 Sensitivity sample preparation

The second stock saliva sample was used to create (in triplicate) 1:5, 1:10, 1:50, 1:100, 1:250, 1:500 and 1:1000 dilutions of saliva in distilled water; α-amylase activities were calculated for the dilutions based on the results from the Phadebas® Amylase Test method. Dilutions were stored on ice until required and vortexed briefly before use. A volume of 20 µL of each diluted sample was deposited onto glass sheets to maximise contact between the sample and Phadebas® paper (all glass sheets referred to herein were decontaminated with a 1:20 TriGene solution and rinsed with distilled water prior to use). Fresh neat saliva and distilled water were used as the positive and negative control, respectively. Wet samples were examined at room temperature and in an incubator at 37°C with Phadebas® paper, following the Phadebas® Forensic Press Test protocol (Magle Life Sciences). This protocol was followed for all subsequent examinations; observations were recorded at 1, 2, 3, 4, 5, 10, 20, 30 and 40 minutes and were assigned a reaction score based on the colour intensity exhibited on the Phadebas® paper.

2.3 Determination of specificity

Saliva, blood, faecal, nasal, perspiration, semen, tear, urine, and vaginal samples were used to create stains by depositing 20 µL of liquid or swabbing the sample (where appropriate) onto glass sheets. Fresh neat saliva and distilled water were used as the positive and negative control, respectively. Stains were left to dry for 24 hours at room temperature to simulate stains encountered in forensic laboratories, before being examined with Phadebas® paper at room temperature and in an incubator at 37°C.

2.4 Detection of saliva in mixed body fluid stains

The third stock saliva sample was used to create body fluid mixtures of saliva:blood, saliva:semen and saliva:urine in ratios of 1:0, 3:1, 1:1, 1:3 and 0:1, in triplicate. Samples were stored on ice until required and vortexed briefly before use. For each sample, 20 µL was deposited onto a glass sheet. Saliva:vaginal secretion stains were also generated by depositing 20, 10, 5, 2 and 1 µL of neat saliva from the saliva sample onto the glass sheet (in duplicate). Vaginal secretion samples, that had been collected simultaneously, were subsequently swabbed over each wet saliva deposit. Fresh neat saliva and distilled water were used as the positive and negative control, respectively. Stains were left to dry for 24 hours at room temperature before being examined with Phadebas® paper, at room temperature.

2.5 Detection of saliva on various substrates

2.5.1 Preparation of substrates

All textile substrates examined in this study originated from pre-owned garments acquired from an opportunity shop. Garment composition was as follows: garment 1 100% neoprene; garment 2 and 5 100% synthetic; garment 3 and 4 100% polyester; garment 6, 100% silk; garment 7 50% angora, 50% lambswool; garment 8–11 100% cotton; garment 12 85% cotton, 10% polyester, 5% elastane; garment 13 95% cotton, 5% elastane; garment 14 55% cotton, 45% nylon.

Garments were washed in a Bosch 7 kg front load washing machine as per the washing instruction label. Synthetic fibre (garment 1–5), natural fibre (garment 8–11) and blended fibre garments (12–14) were washed on the ‘Super15’’ cycle with FAB® Fragrance Temptations™ laundry powder. This laundry powder is known to contain unspecified enzymes, however it was not sampled as a

negative control since α-amylase activity does not persist on textiles laundered under standard wash cycle conditions (Feia & Novroski, 2013). Delicate natural fibre garments (6 and 7) were washed on the 'Delicate/Silk' cycle with Softly® Premium Laundry Liquid Delicates & Woollens. All garments were left to line-dry.

Paper samples were collected from an unopened quire or ream of sheets. Impermeable substrates were decontaminated with a 1:20 TriGene solution and rinsed with distilled water, prior to use. Swatches measuring approximately 100 ×150 mm were excised, in duplicate, for all substrates and were stored at room temperature in separate paper bags until required.

2.5.2 Determination of substrate porosity by saturation

In order to determine substrate porosity (P_t), the total volume (V_t) of each substrate was initially determined. Each substrate was subsequently placed in a known volume of water; the displacement of the water ($\Delta Volume_{H2O}$) was then observed and substituted into equation (1) along with the total volume (V_t) of the substrate. This was repeated for all substrates.

$$Porosity\ (P_t)\ \% = \frac{Pore\ volume\ (V_p)}{Total\ volume\ (V_t)} \times 100, \quad (1)$$

where, V_t is equal to the L × W × H of the substrate, and V_p is equal to V_t - $\Delta Volume_{H2O}$.

The porosity classification of the substrates in this study as defined by equation (1), were as follows: non-porous (0%), semi-porous (>0–10%), and porous (>10%).

2.5.3 Substrate stain preparation

A volume of 20 µL of the fourth stock saliva sample was deposited onto each of the various non-porous, semi-porous and porous substrates; this sample also acted as the positive control. Distilled

water was used as the negative control. Stains were left to dry for 24 hours at room temperature before being examined with Phadebas® paper, at room temperature.

3 Results

Since all observations of Phadebas® paper were manifested as qualitative results (i.e. colour intensity), interpretation was potentially influenced by examiner subjectivity. Thus, results were interpreted by a single examiner based on a devised qualitative scoring system for consistency (Figure 1).

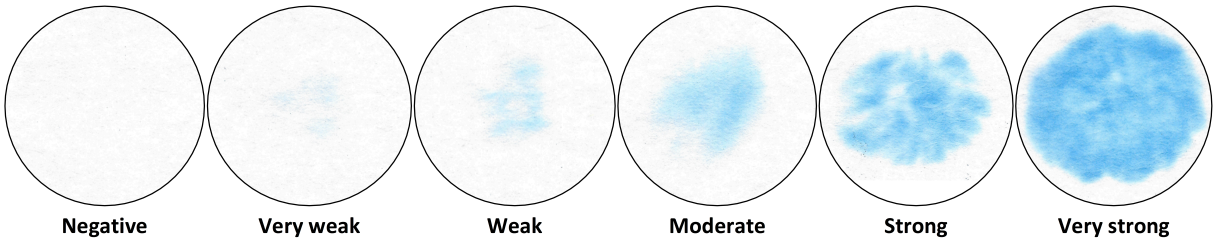

Figure 1: Colour intensity scale for interpreting Phadebas® paper observations. Colour intensity scores were categorised as follows: negative (N); very weak (VW); weak (W); moderate (M); strong (S); very strong (VS).

3.1 Sensitivity of Phadebas® paper

Following the quantification of α-amylase activity in the stock saliva sample (94,988 ± 0.17%), dilutions subsequently produced from the solution were tested by observing the resulting colour reaction intensity on Phadebas® paper, in order to assess the limit of detection. Saliva samples with α-amylase activities of 94,988 ± 0.17% (neat) down to 189 (1:500 dilution) generated a positive result on Phadebas® paper within the 40-minute examination period at both room temperature and 37°C (Table 1). The intensity of the colour reactions produced on Phadebas® paper for all three replicates of each dilution, at both temperatures were highly reproducible. Additionally, the time to observe a positive result increased, as the dilution factor increased (i.e. as the α-amylase activity decreased). Furthermore, the final colour intensity achieved at the end of the 40-minute examination decreased as the dilution factor increased (i.e. as the α-amylase activity decreased).

Table 1: Phadebas® Forensic Press Test results for dilutions of liquid saliva examined at 23°C and 37°C.

Sample (n=6)	α-amylase activity (U/l)	Time of observation (minutes)								
		1	2	3	4	5	10	20	30	40
Positive control	-	M			S			VS		
Negative control	-					N				
Neat	94,988*	M			S			VS		
1:5	18,997	W			M		S		VS	
1:10	9,498	N	VW	W		M			S	
1:50	1,899		N		VW		W		M	
1:100	949			N			VW		W	
1:250	379			N				VW		W
1:500	189			N				VW		
1:1000	94					N				

Colour intensity score: N = negative; VW = very weak; W = weak; M = moderate; S = strong; VS = very strong

*Mean of 3 measurements, error margin ± 0.17%

-Samples of unknown α-amylase activity

3.2 Specificity of Phadebas® paper

Saliva and other forensically relevant body fluid stains were tested by observing the intensity of the resulting colour reaction produced on Phadebas® paper, to assess cross-reactivity. The results obtained for saliva (n=8), blood (n=1), faecal (n=8), nasal (n=8), perspiration (n=4), semen (n=5), tear (n=2), urine (n=8) and vaginal (n=2) samples with Phadebas® paper, at 23°C versus 37°C, are summarised in Table 2. Phadebas® paper was able to detect α-amylase activity in all saliva samples 1 minute into the examination, at both temperatures; strong/very strong positive results were exhibited at 5 minutes for all saliva samples. Positive results were generated for faeces at both temperatures between 10 and 40 minutes. Nasal secretions produced mainly negative results, but a very weak result was observed at 40 minutes (23°C), and a moderate result was produced at 20 minutes (37°C). Similarly, perspiration, semen and urine exhibited mostly negative results, but produced a positive indication for α-amylase activity at 37°C between 20 and 40 minutes. All blood, tear fluid and vaginal secretion samples resulted in negative observations.

Due to a concern that an adequate amount of biological material was not transferred during sample deposition for samples collected using swabs, swabs were also dried for 24 hours at room temperature and were directly examined with Phadebas® paper, at room temperature (data not shown). For this examination, 100% of faecal, nasal and tear fluid swabs returned positive results, observed at 10, 3 and 30 minutes into the examination, respectively. Vaginal swabs returned 100% negative results for α-amylase activity.

Table 2: Phadebas® Forensic Press Test results for saliva and other forensically relevant body fluids examined at 23°C and 37°C.

Sample		23°C									37°C								
		Time of observation (minutes)									Time of observation (minutes)								
		1	2	3	4	5	10	20	30	40	1	2	3	4	5	10	20	30	40
Control	Positive	M			S		VS				M			S			VS		
	Negative						N									N			
Saliva	1	M			S		VS				S						VS		
	2	W		M	S		VS				W		M	S			VS		
	3	W	M			S		VS			W	M		S			VS		
	4				S				VS				S				VS		
	5	M			S		VS				M			S			VS		
	6			S			VS						S				VS		
	7, 8	M			S		VS				M		S				VS		
Blood	1						N*									N*			
Faeces	1			N†			VW†	W†	M†	S†			N				W	M	S
	2			N†				VW†		W†					N†				VW†
	3						N									N			
	4				N				VW				N				VW		M
	5				N†				VW†				N†				VW†		W†
	6, 7			N†			VW†		M†				N†				W†	M†	S†
	8				N†				VW†	W†			N†				VW†	W†	S†
Nasal secretions	1, 2, 4-7						N									N			
	3						N						N				M		
	8					N				VW						N			
Perspiration	1						N‡									N‡			
	2						N‡								N‡				VW‡
	3, 4						N‡						N‡				VW‡	W‡	M‡
Semen	1						N§					N§						VW§	W§
	2						N§									N§			

	3, 5	N[§]	N[§]		VW[§]
	4	N[§]	N[§]		W[§]
Tear fluid	**1, 2**	N	N		
Urine	**1, 2, 4-8**	N[¶]	N[¶]		
	3	N[¶]	N[¶]	VW[¶]	W[¶]
Vaginal secretions	**1, 2**	N	N		

Colour intensity score: N = negative; VW = very weak; W = weak; M = moderate; S = strong; VS = very strong
[*]Red-brown stain visible
[†]Green-brown stain visible
[‡]White area visible
[§]Clear area visible
[¶]Yellow stain visible

Blood, faeces, perspiration, semen and urine stains exhibited colour transfers to Phadebas® paper (Figure 2). Blood developed an intense red-brown stain, while the positive reaction for α-amylase activity in faeces was typically accompanied by a green-brown stain. Perspiration displayed a white area, which was devoid of visibility of the blue starch polymer-dye complexes (as observed in the negative control). Semen was observed as a clear area, and urine transferred a faint yellow colouration to the Phadebas® paper.

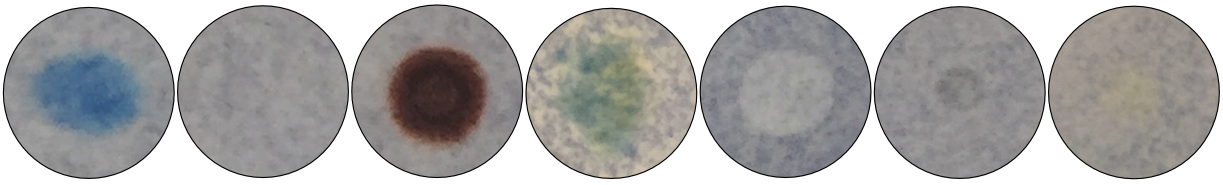

Figure 2: Colour transferred from blood, faecal, perspiration, semen and urine stains on Phadebas® paper. Phadebas® paper exhibited red-brown staining in the presence of blood, green-brown staining in the presence of faeces, a white area in the presence of perspiration, a clear area in the presence of semen, and, yellow staining in the presence of urine. Positive and negative controls are also shown for reference.

3.3 Body fluid interference

To examine the possible interference of saliva detection in the presence of accompanying body fluids, mixed saliva:body fluid stains were tested by observing the resulting colour reaction intensity produced on Phadebas® paper. Results are summarised in Table 3. Despite being

accompanied by a red-brown stain, α-amylase activity, in all stains consisting of saliva and blood, was detectable with Phadebas® paper. Compared to the positive control, the time to elicit any positive reaction was longer for saliva:blood stains at all ratios, especially the 1:3 stains, which only reached a moderate final colour intensity. The time required to achieve results of higher intensity increased as the ratio of saliva:blood decreased, which was expected due to the increasing dilution of saliva. This was similarly seen in the saliva:semen stains. α-Amylase activity in the saliva:semen stains was also detectable at all ratios with Phadebas® paper. Saliva in urine produced similar results to the positive control for the 3:1 and 1:1 stains; a negative result was obtained for the 1:3 saliva:urine stain. Due to this unusual observation, a secondary examination for saliva:urine was undertaken with an alternative urine sample; all stains examined in this secondary test produced false negative results after the 40 minute examination with Phadebas® paper. Saliva:vaginal secretion mixtures displayed highly variable results. Due to a concern that an adequate amount of vaginal fluid was not mixed with the saliva deposits, swabs used to generate the saliva:vaginal secretion stains were allowed to dry for 24 hours at room temperature and were directly examined with Phadebas® paper, at room temperature; similar highly variable results were observed (data not shown).

Table 3: Phadebas® Forensic Press Test results for saliva:body fluid mixtures.

Sample		Time of observation (minutes)								
		1	2	3	4	5	10	20	30	40
Control (n=3)	**Positive (1:0)**	M	S			VS				
	Negative					N				
Saliva: Blood (n=3)	**3:1**	N*		W*		M*	S*		VS*	
	1:1	N*		VW*		W*	M*	S*	VS*	
	1:3		N*			VW*	W*		M*	
	0:1					N*				
Saliva: Semen (n=3)	**3:1**	W†		M	S			VS		
	1:1	W		M		S			VS	
	1:3	VW	W			M	S		VS	
	0:1					N†				
Saliva: urine‡ (n=3)	**3:1**	M		S				VS		
	1:1	W	M		S			VS		

	1:3									N											
	0:1									N											
	20 µl:swab 1				N					VW				W				M			S
	20 µl:swab 2	W					M						S				VS				
	10 µl:swab 3	N		VW					W							M					
	10 µl:swab 4	VW				W						M						S			
	5 µl:swab 5						N					VW				W					
Saliva:vaginal secretions	**5 µl:swab 6**		N					VW				W				M				S	
	2 µl:swab 7			N					VW			W		M			S				
	2 µl:swab 8			N						VW					W						
	1 µl:swab 9			N						VW					W						
	1 µl:swab 10		N					VW				W				M					
	Swab 11									N											
	Swab 12									N											

Colour intensity score: N = negative; VW = very weak; W = weak; M = moderate; S = strong; VS = very strong

*Red-brown stain visible

†Clear area visible

‡A secondary test using a different urine sample produced negative results for all ratios

3.4 Influence of substrate porosity

To assess if substrate porosity influenced the detection of saliva, neat saliva stains deposited on various non-porous, semi-porous and porous substrates were tested by observing the intensity of the colour reaction produced on Phadebas® paper. Time to achieve a strong positive result on Phadebas® paper was plotted against porosity for each substrate to obtain a trend line equation ($y = 0.2117x + 6.381$) and a coefficient of determination (R^2) value (0.29798) (Figure 3). The R^2 value indicated that the time to achieve a strong positive result with Phadebas® paper was weakly dependent upon substrate porosity. In general, non-porous substrates achieved a strong positive result more quickly, compared to semi-porous and porous substrates. Positive indications for α-amylase activity were achieved in the 40-minute examination period for all substrates tested. However, saliva stains on garment 11 and 12 failed to produce a strong positive final colour intensity wit Phadebas® paper within the 40-minute examination period, producing very weak and moderate results, respectively.

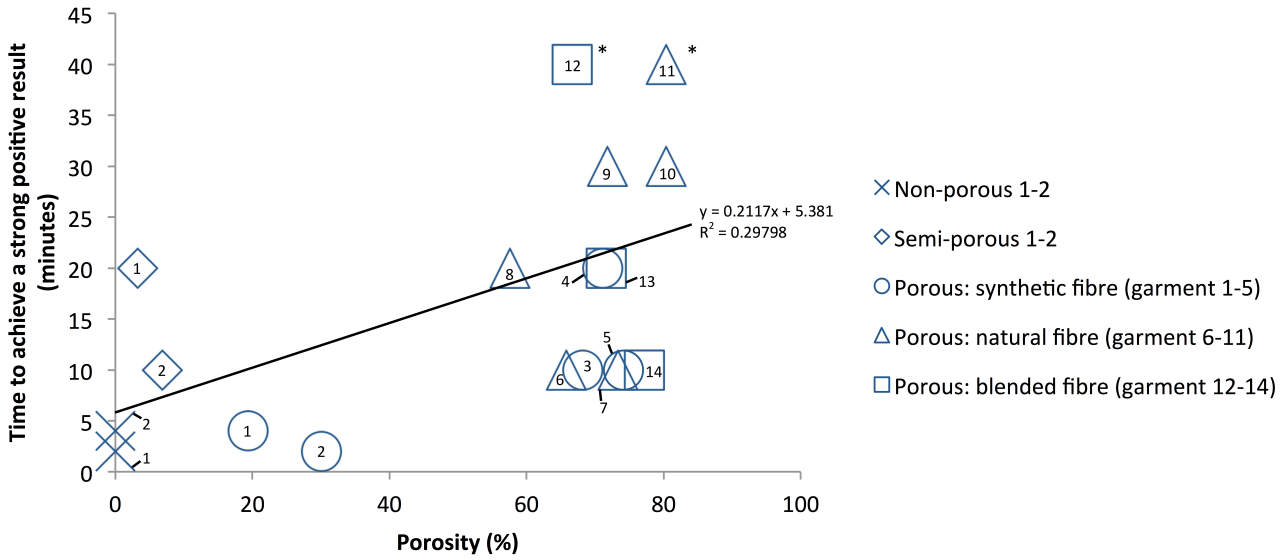

Figure 3: Influence of substrate porosity (%) on the time to achieve a strong positive reaction with Phadebas® paper. Substrates that did not achieve a strong final colour intensity within the 40-minute examination period, but produced a positive result are indicated by (*).

4 Discussion

The experiments undertaken in this study have identified the limitations of Phadebas® paper for the purpose as a presumptive screening tool for saliva during forensic examinations. In particular the sensitivity, specificity, effects of temperature on sensitivity and specificity, ability to detect saliva (α-amylase activity) in mixed samples, and the effects of substrate porosity on the ability to detect saliva have been effectively assessed. Presumptive screenings tools are ideally, highly sensitive, specific and produce minimal false negative results (Houck & Siegal, 2011). This study has identified Phadebas® paper to be much more sensitive than what is currently believed; the manufacturer implies that the lower detection limit of Phadebas® paper for α-amylase activity is 2000 U/L (Magle Life Sciences, 2014). However, it has been determined here that an α-amylase activity of 189 U/L could be detected. Previous studies that have attempted to validate the sensitivity of Phadebas® paper have typically presented the lower detection limit as a dilution factor, neglecting any consideration for the initial α-amylase activity of the sample (Breathnach & Moore, 2013; Davidek, Unpublished results; Hedman et al., 2008; Park et al., 2015; Roda et al., 2014). As a result, it has been reported that Phadebas® paper can detect α-amylase activity in saliva samples within a wide range of dilutions, between 1:100–1:1000; the value determined in this study (1:500) is within this range. This large variation in reported values may be explained by the existence of intra- and inter- personal variations of α-amylase activity in the population due to a number of factors, such as genetic influences (Perry et al., 2007), age (Ben-Aryeh et al., 1986), drug use (Enemchukwu, Ubaoji, Igwilo, & Udedi, 2013; Maruyama et al., 2003), illness (Kazmierczak, 1997), diurnal rhythm (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007), stress (Nater et al., 2006) and food intake (Tsutsumi et al., 1991). An additional factor that may contribute to this variation is the decision to use dried/aged samples rather than fresh in such

experiments; α-amylase activity has been shown to decrease to approximately 17% of the initial activity of the sample during a drying period of 24 hours (Tsutsumi et al., 1991) and to 1% of the original α-amylase activity after 49 days (Gutowski & Henthorn, 1983). By quantifying the α-amylase activity in the sample prior to examination and analysing a wet sample, this study has provided significance to the reported dilution factor and has negated any drying/ageing effects on α-amylase activity.

The specificity of Phadebas® paper is influenced by its sensitivity. The manufacturer states that to assure selectivity for saliva, it must not detect stains with α-amylase activity below 2000 U/L in the 40-minute examination and that no other forensically relevant body fluid (other than faeces) will react within 10 minutes following the current protocol (Magle Life Sciences, 2014). Many studies have previously identified that other body fluids including blood, breast milk, faeces, nasal secretions, perspiration, semen, tears and urine can contain α-amylase activities that exceed the lower sensitivity threshold determined in this study (approximately 189 U/L) compared to the previously accepted value (2000 U/L), which theoretically means that Phadebas® paper should only exhibit cross-reactivity with faeces, tears and urine (Akutsu et al., 2010; Auvdel, 1986; Gutowski & Henthorn, 1983; Kipps & Whitehead, 1975; Tsutsumi et al., 1991; Willott, 1974). This work has demonstrated that other forensically relevant body fluids including faeces, nasal secretions, perspiration, semen, tear fluid and urine have the potential to exhibit cross-reactivity when examined with Phadebas® paper and in addition to faeces, nasal secretions also have the potential to react with Phadebas® paper within 10 minutes. It should be noted that if this study were to be repeated, directly examining samples collected with swabs would be used in preference to swabbing the samples onto glass. In the literature there are conflicting findings surrounding the ability of other forensically relevant fluids to exhibit cross-reactivity with Phadebas® paper; typically, such results have not been observed with other forensically relevant body fluids, with the

exception of faeces (Breathnach & Moore, 2013; Watchman et al., 2008). However, none of these studies examined nasal secretions or tear fluid. Although the sample size in this this study was relatively small, similar results were obtained to that of Olsén et al. (2011) and Davidek (Unpublished results), which demonstrated cross-reactivity with perspiration, semen, urine and vaginal secretions. It is to be noted that breast milk was not examined in this study due to difficulty in obtaining such a sample.

In this study, other forensically relevant body fluids such as blood, faeces, perspiration, semen and urine were typically associated with a colour transfer from the stain to Phadebas® paper, allowing some degree of stain origin discrimination. However, at least one faecal sample and all nasal secretion samples reacted with Phadebas® paper without being accompanied by a colour transfer, akin to saliva stain interactions with Phadebas® paper. Thus, it is recommended that positive results on Phadebas® paper should not be assigned a class of origin based on the Phadebas® Forensic Press Test alone. To further enforce this recommendation, it has been shown in two studies (Breathnach & Moore, 2013, 2015), that only a rather small proportion (13%) of items that exhibit positive indications for saliva with Phadebas® paper, return positive results with the confirmatory test Rapid Stain Identification Saliva (RSID™-Saliva).

It is apparent from the sensitivity study that analysing saliva stains with Phadebas® paper at the optimum temperature for α-amylase activity (i.e. body temperature, 37°C) does not increase sensitivity. Conversely, a study by Hedman et al. (2008) demonstrated that the use of an incubator (at 37°C) with Phadebas® paper, resulted in a higher sensitivity of Phadebas® paper to the sample; a dilution of 1:200 could be detected at 37°C, as opposed to 1:100 at room temperature, though with low reproducibility. However, in the specificity study of this work, examination at the higher temperature appeared to increase the incidence of cross-reactivity. This may be explained due to

the use of dried samples as opposed to wet samples used in the sensitivity study. Dried stains, in the presence of distilled water (used to moisten the exhibit and Phadebas® paper in the current protocol), may take some time to become incorporated into a liquid again, which is required for α-amylase activity to be detected using Phadebas® paper. Due to an increase in kinetic energy of the molecules in the stains at a higher temperature (37°C), the recovery of α-amylase activity may be achieved more rapidly, allowing the α-amylase in the stains to react with Phadebas® paper for a longer period of time. This may give a false indication of an increase in sensitivity and thus result in apparent enhanced cross-reactivity. It is known that some forensic laboratories have adopted changes in the Phadebas® Forensic Press Test protocol regarding the examination temperature by increasing it to 37°C (Skrinnikoff, Donnelly, & Silenieks, 2016). By implementing this change in examination temperature, there appears to be a trade-off between sensitivity and specificity. It is therefore recommended that to maintain specificity for saliva no modifications regarding examination temperature should be made to the current protocol.

This work found that for saliva in mixed body fluid stains, only semen did not adversely interfere with the detection of saliva with Phadebas® paper. In the samples where blood was the major component of the saliva:blood mixture, the overall observation on Phadebas® paper was substantially weaker than expected. Whole blood has been shown in one particular study to interfere with α-amylase activity in saliva; this was thought to be due to the high protein content (Tsutsumi et al., 1991), which may provide an explanation for the results obtained in this study. Variable reactions were observed with saliva in urine and vaginal secretion mixtures. It can be suggested that for these mixtures, perhaps the pH of the accompanying fluid may decrease α-amylase activity as a result of enzyme denaturation. It has previously been reported that the optimal pH for the activity of α-amylase (in vitro) is 7.0 (Rudeekulthamrong & Kaulpiboon, 2012). The pH of blood is tightly regulated, between pH 7.35–7.45 (Stanfield, 2011); the normal pH range

of semen is 7.2–7.8 (World Health Organization, 2010); urine can widely range from pH 4.6–8.0 (Riley & McPherson, 2017); and, vaginal secretions typically have a pH of 3.8–4.5 (Kelly, 1990). Further supporting this idea is the fact that, despite urine having the next highest average amount of α-amylase activity, other than saliva or faeces (Kipps & Whitehead, 1975), the urine samples examined in this study reacted less frequently when compared to semen, which is typically more neutral than urine.

No studies prior to this have investigated the potential influence of substrate porosity on the detectability of α-amylase activity with Phadebas® paper. This study demonstrated that the detection of saliva (or α-amylase activity) with Phadebas® paper is affected by the substrate upon which it is deposited. However, substrate porosity appeared to only be a weak predictor for the time to achieve a strong positive result. Other factors that may influence the detectability of saliva is the composition, thickness and construction (for textiles) of the substrate. α-Amylase activity on the most porous substrate tested in this work (originating from garment 11, approximately 84% porous), was not detectable on Phadebas® paper until 40 minutes had elapsed, and even then produced only a very weak result, compared to saliva deposited on less-porous and non-porous substrates, where α-amylase activity was detectable within 1 minute. This also suggests that substrates of very high porosity may produce false negative results with Phadebas® paper. Additionally, many previous studies investigating the sensitivity and specificity of Phadebas® paper deposit saliva samples onto cotton substrates without acknowledging the possible implications of doing so; the results obtained for these studies may be distorted due to the choice of substrate used for sample deposition (Breathnach & Moore, 2013; Davidek, Unpublished results; Hedman et al., 2008; Olsén et al., 2011; Roda et al., 2014; Watchman et al., 2008).

5 Conclusion

Phadebas® paper is more sensitive to α-amylase activity and less specific for saliva (especially when using an examination temperature of 37°C) than has previously been reported. Since the status of saliva stains, and their associated α-amylase activities, are unknown when encountered in forensic situations, Phadebas® paper does not possess the qualities required to accurately identify saliva. Similar results may be observed for stains derived from other body fluids and dilute (or potentially, aged) saliva stains. Furthermore, mixed body fluid samples and porosity of the substrate may potentially influence the occurrence of false negative results. Hence, classification of stain origin should not be based on the results obtained from the Phadebas® Forensic Press Test, that is; a positive reaction for α-amylase activity on Phadebas® paper should not be considered indicative for the presence of saliva. The importance of utilising confirmatory tests, such as RSID™-Saliva following the Phadebas® Forensic Press Test, is recommended, since inclusion of information derived from results from this test alone could potentially lead to incorrect and misleading inferences in forensic investigations. Despite this, Phadebas® paper is useful as a screening tool and may rival the use of ALS techniques for the screening of saliva. In future studies, comparative analyses of Phadebas® paper and ALS should be conducted to determine the method that is most accurate and cost-effective, and least destructive to the sample, for use in forensic laboratories. The detectability of aged stains with Phadebas® paper also needs to be investigated.

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Conflict of interest

The authors declare no conflicts of interest.

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